MOLECULAR AND CELLULAR BIOLOGY, May 1988, p. 2082–2088
0270-7306/88/052082-07$02.00/0

Vol. 8, No. 5

Infrequent Genomic Rearrangement and Normal Expression of the Putative RB1 Gene in Retinoblastoma Tumors

AUDREY D. GODDARD,[1,2] HANNA BALAKIER,[2] MARC CANTON,[2] JAMES DUNN,[1,2] JEREMY SQUIRE,[1,2] EVANGELICA REYES,[2] ANDREW BECKER,[1] ROBERT A. PHILLIPS,[1,2] AND BRENDA L. GALLIE[1,2]*

University of Toronto[1] and The Hospital for Sick Children Research Institute,[2] 555 University Avenue, Toronto, Ontario, Canada M5G 1X8

Received 4 November 1987/Accepted 4 February 1988

Retinoblastoma (RB) tumors develop when both alleles of a gene (RB1) are mutated and unable to function normally. Recently, Friend et al. [S. H. Friend, R. Bernards, S. Rogelj, R. A. Weinberg, J. M. Rapaport, D. M. Albert, and T. P. Dryja, Nature (London) 32:643–646, 1986] reported the cloning of a gene, 4.7R, with some properties expected for the RB1 gene, namely, a high frequency (30%) of genomic rearrangements in tumors and absence of message in all RB tumors examined. To extend the characterization of this gene, we used 4.7R probes to search for genomic rearrangements of DNA and to study the expression of the 4.7R gene in RB tumors, osteosarcoma (OS) tumors arising in RB patients, and other normal and malignant tissues. In 34 previously unreported RB and OS tumors arising in RB patients, we observed only four (12%) with genomic abnormalities. Transcripts of 4.7R were present in 12 of 17 RB tumors, 2 of 2 OS tumors, and all non-RB tumors and normal tissues tested. We were unable to confirm the high frequency of truncated messages of 4.7R in RB tumors reported by Lee et al. (W. H. Lee, R. Bookstein, F. Hong, L. J. Young, J. Y. Shaw, and E. Y. Lee, Science 235:1394–1399, 1987) and Fung et al. (Y. K. Fung, A. L. Murphree, A. Tang, J. Qian, S. H. Hinrichs, and W. F. Benedict, Science 236:1657–1661, 1987) but did confirm the presence of a truncated transcript in the RB cell line Y79. Of the RB and RB-related OS tumors which appeared normal on Southern blots, 2 of 16 or 12% had abnormal transcripts, giving a combined frequency of 22% abnormalities in the 4.7R gene detectable by Southern and Northern (RNA) blot analyses.

Retinoblastoma (RB) tumors arise when embryonic retinal cells acquire mutations in both alleles of a gene, RB1, located in chromosome band 13q14 (4, 16). The presence of tumors with homozygous deletion of the region indicates that tumors arise when this gene is not expressed. In some patients, one of the mutations is carried as a germ line mutation, and in their families, RB is inherited as an autosomal dominant trait. Patients with a germ line mutation in the RB1 locus are also at increased risk for the development of osteosarcoma (OS) tumors (1, 7, 17). Recently, Friend et al. (12) reported the isolation of a human cDNA gene probe (p4.7R) with properties consistent with those of the RB1 gene: expression in some normal and transformed tissues but not in any RB or OS tumors tested and deletion or rearrangement in the genomic DNA of 30% of RB and OS tumors.

Using an identical strategy, Lee et al. (20) and Fung et al. (13) subsequently isolated the same gene and confirmed the same high frequency of genomic rearrangements reported by Friend et al. (12). However, Lee et al. (20) detected transcripts of the 4.7R gene in some tumors, in all instances shorter than the 4.7-kilobase (kb) transcript detected in fetal retina and other normal tissues; Fung et al. (13) reported similar findings. To test the relationship of 4.7R to RB1, we studied DNA and RNA from RB tumors and OS tumors from RB patients. Using the probe isolated by Friend et al. (12), we found rearrangement of this gene in a small number of RB and OS tumors. However, we were unable to confirm the presence of truncated transcripts in tumors; most tumors contained a normal-size transcript.

* Corresponding author.

MATERIALS AND METHODS

Tumor specimens. Twenty RB tumors were studied initially as surgical specimens, obtained at the time of surgical enucleation for treatment of RB. Twenty RB tumors were studied as cell lines that were grown from surgical specimens in our laboratory, both in the anterior chamber of the eyes of athymic nude mice (14) and in tissue culture, initially on feeder layers (15). Subsequently, the suspension RB cell lines have been grown in Dulbecco medium with 15% fetal calf serum, 10 μg of bovine insulin per ml, and 5×10^{-5} M 2-mercaptoethanol. Eight RB tumors were studied both as surgical specimens and as tissue culture cell lines.

Fourteen of the RB tumors were from individuals who were unilaterally affected and presumed to have no constitutional mutation at the RB1 locus. The other 18 RB tumors were obtained from 14 patients assumed to carry germ line mutations at the RB1 locus because they were bilaterally affected and/or had a family history of RB. RB tumors were obtained from both eyes of four bilaterally affected individuals and were shown by karyotype to be separate primary tumors (30). To confirm that the tumors then growing were not contaminated with other cells, repeat karyotype determinations were performed. The tumors had retained the characteristic patterns of markers previously reported (30).

In addition, three RB tumor cell lines, Y79 (26), WERI-RB1 (24), and RB355 (27, 30), for which data on the p4.7R gene have been previously described (8, 9, 12, 13, 20), were studied. These lines were maintained as suspension cultures, as described above. DNA was available from two previously reported tumors, LA-RB69 (2) and #9 (9).

Two OS tumor cell lines were developed from individuals who were previously afflicted with bilateral RB (their RB tumors were not available for study) and who, therefore, carry germ line mutations at the RB locus (17). Other control

tumors included a myelogenous leukemia line, HL60 (33); a neuroblastoma line, IMR32 (28); adenovirus type 5- and adenovirus type 12-transformed human fetal retinal lines (3); and a new primitive neuroectodermal tumor line, Tab-1 (A. Keating, unpublished data).

Normal tissues. The following normal tissues were obtained from postmortem examinations: fetal retina; retina, spleen, liver, brain, and kidney from a newborn; and brain, liver, and testis from an adult. Lymphoblastoid cell lines were obtained by Epstein-Barr virus (EBV) transformation of peripheral blood lymphocytes (34).

DNA and RNA preparations. DNA was extracted from 5×10^7 to 1×10^8 cells by the standard method of phenol and isoamyl alcohol extraction, followed by ethanol precipitation (23), or by removal of DNA from guanidine thiocyanate-cesium chloride gradients (5) used to prepare total cellular RNA, followed by standard phenol and isoamyl alcohol extraction and ethanol precipitation. Poly(A)$^+$ RNA was obtained by oligo(dT)-cellulose chromatography (23).

Probes. The p4.7R cDNA probe (a gift from T. Dryja) contains two *Eco*RI fragments, of approximately 3.8 and 0.9 kb. These *Eco*RI fragments were prepared by isolation from the plasmid and are referred to as the 3.8 and 0.9 probes, respectively. Other probes from the 4.7R gene, p7H30.7R, p7RV1.55L, and p23RI1.02, were a gift from T. Dryja. The 0.6-kb ED1 fragment of the esterase D (ESD) gene (31) and the N-*myc* probe (28) pNB-1 (a gift from J. M. Bishop) were purified from plasmids; all probes were labeled with [α-^{32}P]dCTP by random-primer labeling (11) to a specific activity of 5×10^8 to 1×10^9 cpm/μg.

Southern blots. DNA samples (5 to 7 μg) were digested with restriction endonucleases (*Hin*dIII, *Bam*HI, *Sac*I, *Eco*RI, *Pst*I, *Acc*I, *Nci*I, and *Kpn*I) under conditions recommended by the supplier (Pharmacia [Canada] Inc. or Boehringer Mannheim Canada). Digested genomic DNA was separated by gel electrophoresis in 1.0 or 0.8% agarose (SeaKem GTG), and blots were made onto nitrocellulose (29) (Schleicher & Schuell, Inc.) or Zetaprobe (25) (Bio-Rad Laboratories) membranes. Hybridization to [α-^{32}P]dCTP-labeled probes was carried out in 4× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M sodium phosphate dibasic, and 0.001 M EDTA)–0.5% Carnation instant milk–1% sodium dodecyl sulfate 10% dextran sulfate–50% formamide–1 mg of yeast RNA per ml at 42°C. After hybridization, filters were washed under high stringency conditions (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% sodium dodecyl sulfate, 65°C). DNA hybridization signals were estimated by band density on autoradiograms by using a Bio-Rad 620 Video Densitometer and Bio-Rad Model 3392A Integrator.

Northern (RNA) blot analysis. Between 3 and 9 μg of poly(A)$^+$ RNA, obtained by oligo(dT)-cellulose chromatography (23), was loaded per lane, separated by electrophoresis through a 1% agarose gel (23), and transferred to GeneScreen (New England Nuclear Corp.) under recommended conditions (6). The 3.8 and 0.9 probes were hybridized and washed under conditions described previously (32). Subsequent or simultaneous hybridization with ED1 was an internal control for the presence of intact mRNA. Filters were exposed for 40 to 64 h at −70°C with an intensifying screen.

RESULTS

Genomic rearrangements of the 4.7R gene in tumors. We examined five previously reported tumors, WERI-RB1 (12, 20), Y79 (12, 13, 20), #9 (12), LA-RB69 (13), and RB355 (20), for genomic rearrangements in the 4.7R and ESD genes. The two RB tumors (WERI-RB1 and #9) reported (12, 20) to be totally deleted for the 4.7R gene were confirmed to be deleted with the 3.8 probe (Fig. 1a). Tumor #9 retained the 16-kb band noted by Fung et al. (13) at the 5′ end of the cDNA.

Both the ESD gene (10, 31) and 4.7R map within chromosome band 13q14, but no rearrangements have been detected in RB tumor DNA samples with the ESD gene probe (19, 31). Analysis of signal intensities from WERI-RB1 DNA with probes for 4.7R, the ESD gene, and N-*myc* confirmed the homozygous deletion of 4.7R and showed a hemizygous deletion for the ESD gene (data not shown). Our karyotype of WERI-RB1 showed only one apparently normal chromosome 13, which must contain a submicroscopic deletion removing the 4.7R gene but not affecting the ESD locus. The other tumor deleted for most of 4.7R, #9, had normal genomic levels of the ESD gene. Surprisingly, tumor LA-RB69, inferred to have a submicroscopic deletion including both the ESD and RB1 gene loci in the one chromosome 13 retained in the tumor (2), appeared entirely normal when probed with p4.7R (Fig. 1b). LA-RB69 also had a normal ESD genomic pattern, in agreement with the conclusions drawn by Lee et al. (21), based on the detection of ESD protein with antibody and low (15% of normal) levels of enzyme activity. Thus, apparently a mutation in 4.7R, which does not alter the gross genomic structure, arose independently of an ESD gene mutation which reduced the enzyme activity of the protein produced from this allele in the tumor. RB355 also appeared normal when tested with the 4.7R and ESD gene probes (data not shown). The patterns observed with eight restriction enzyme digests of the other previously published (12, 13, 20) RB tumor, Y79, were also normal (Fig. 1a and data not shown).

We examined 34 previously unreported tumors by Southern blotting after digestion with several restriction enzymes: *Hin*dIII (Fig. 1a), *Bam*HI, *Kpn*I, *Eco*RI, and *Pst*I. The blots were hybridized with the 3.8 and 0.9 probes. Normal band patterns were observed in 28 of 32 RB tumors and in 2 of 2 OS tumors from RB patients.

Two tumors, RB530 and RB537, had homozygous deletions affecting all bands recognized by p4.7R (Fig. 2). Karyotype analysis of RB530 showed a small intestinal deletion of 13q14 in the only detectable chromosome 13. Because of its slow growth rate, we were unable to karyotype RB537.

Only two RB tumors had rearrangements within the 4.7R gene. The relative intensities of the bands in the *Hin*dIII digests of the RB tumors were quantified by densitometry. Similar band intensity profiles were obtained for all but two of the nondeleted RB tumors, RB414 and RB369E (Fig. 1a). In contrast to the normal pattern of higher intensities for bands 1 and 3 (9.4 and 6.2 kb, respectively) obtained with the 3.8 probe, both of these unilateral tumors had equal intensities of the largest four bands, suggestive of a hemizygous deletion of the 9.4 - and 6.2-kb bands; the small 2.0-kb band was also reduced in intensity. According to the gene structure presented by Lee et al. (20), these three fragments are contiguous at the 3′ end of the gene. Further analysis of RB414 with six other restriction enzymes (*Acc*I, *Bam*HI, *Eco*RI, *Kpn*I, *Pst*I, and *Sac*I) showed analogous band intensity changes with the 3.8 probe. Although no junction fragments indicative of a deletion were observed, we tentatively conclude that RB414 has a partial hemizygous deletion involving the 3′ end of one of the two 4.7R alleles.

RB369E, however, had the most convincing evidence for

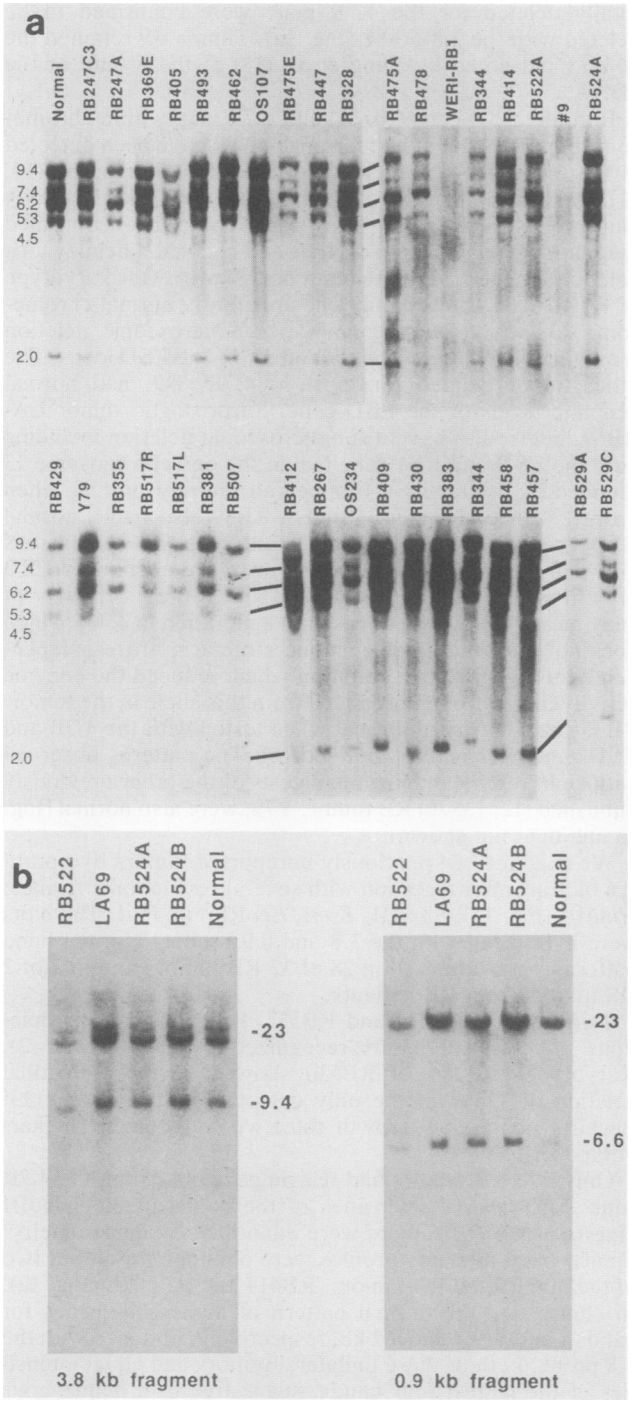


FIG. 1. (a) Southern blot analysis of 33 RB and 2 OS tumor DNA samples digested with *Hin*dIII and hybridized to the 3.8 probe. Tumors WERI-RB1 and #9 show deletion; RB369E and RB414 show a relative decrease in the intensities of the 9.4-, 6.2-, and 2.0-kb bands, suggesting hemizygous 3′ deletion of the 4.7R gene in these tumors. (b) Southern blot of *Kpn*I-digested DNA from LA-RB69 and two other RB tumors (RB524A and RB524B are one tumor) and normal DNA hybridized with the 0.9 and 3.8 probes. The uppermost band in the 3.8 panel is residual signal left from a previous probing. The numbers in the margins are sizes in kilobases.

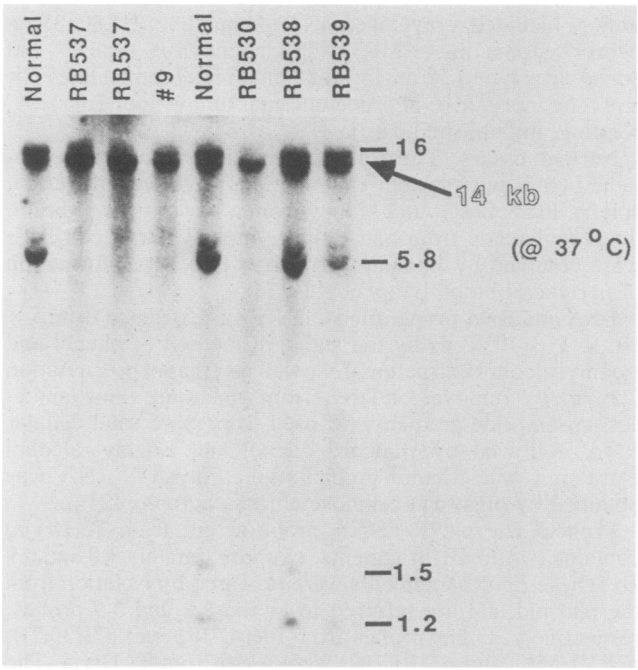


FIG. 2. Demonstration of a cross-hybridizing sequence. Southern blot of *Hin*dIII-digested DNA shows a 14-kb band in all samples after hybridization to the 0.9 probe at 37°C that was not seen at 51°C (results not shown). RB538 and RB539 contain all the expected bands, at 16, 5.8, 1.5, and 1.2 kb. RB530 and RB537 are totally deleted for all bands, except the cross-hybridizing 14-kb band. Tumor #9, on the other hand, retains the 16-kb band, suggesting that the deletion in this tumor does not include the 5′ end of the gene.

both deletion and rearrangement of the 4.7R gene. *Bam*HI, *Sac*I, *Eco*RI, and *Acc*I digestion of DNA resulted in normal patterns of bands with both of the 4.7R probes. A 10-kb *Pst*I band present in the constitutional DNA when probed with the 3.8 fragment of p4.7R was missing from the tumor (data not shown). These data, in conjunction with the reduced

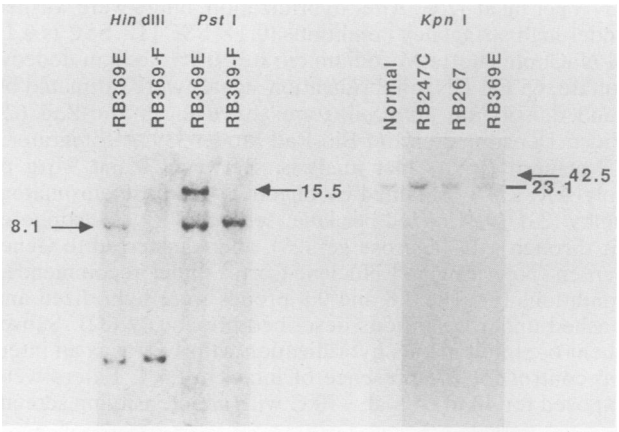
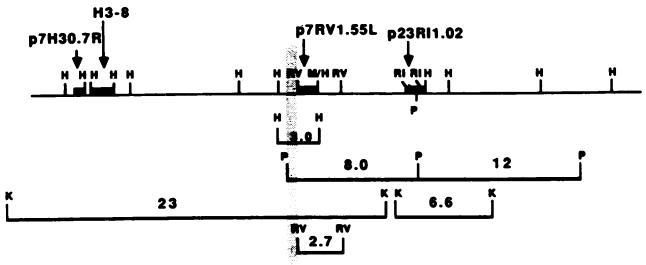


FIG. 3. Demonstration of a rearrangement in RB369E. Fibroblast and tumor DNA from patient 369, normal unrelated DNA, and DNA from two unrearranged tumors (RB247C and RB267) were digested with the indicated restriction enzymes and probed with p7RV1.55L, a genomic sequence from a 5′ intron of 4.7R. Fragments unique to the tumor RB369E are marked with arrows. Sizes are shown in kilobases.

intensity of the *Hin*dIII bands containing the three most 3′ exons (Fig. 3), are suggestive of a heterozygous deletion at the 3′ end of one allele of 4.7R.

RB369E also showed a genomic abnormality in the 5′ portion of the gene. Novel fragments were observed with two genomic probes from 4.7R intron sequences, p7RV 1.55L and p23RI1.02 (9) (Fig. 3 and 4), and with the 0.9 probe. Junction fragments were observed in *Hin*dIII, *Pst*I, and *Kpn*I digests of RB369E DNA by using p7RV1.55L (Fig. 3). The p7RV1.55L and p7H30.7R (9) probes hybridized to the normal *Kpn*I 23-kb fragment, as well as to the 42.5-kb junction fragment observed in a *Kpn*I digest. The sizes of the *Kpn*I fragments were determined by field inversion gel electrophoresis. These observations were most likely the result of an insertion into the area shaded in Fig. 4. The abnormalities (Fig. 4) are consistent with a 19-kb insertion into an intron which appears to exist between the fragments detected with the 3.8 and the 0.9 probes.

Ubiquitous expression of the 4.7R gene in normal tissues and most tumors. To examine expression of the 4.7R gene, poly(A)$^+$ RNA was hybridized with the 3.8 and ED1 probes (Fig. 5); the uniform expression of the ESD gene in all tissues makes it a useful internal control for studies of expression (32). In 12 of 17 previously unreported RB tumors and 2 of 2 OS tumors from RB patients (OS108 and OS234), an apparently normal 4.7R transcript was detected with the 3.8 probe. RB530 and RB537 (data not shown), shown to have total deletions of 4.7R, and RB369E (Fig. 5a), with probable rearrangement of both alleles, lacked detectable transcripts. Unfortunately, RNA was not available from RB414, a tumor with a putative heterozygous 3′ deletion. RB383, which had a normal genomic pattern with all probes and restriction enzymes tested, gave a very faint signal with



ENZYME	p7RV1.55L		p7RI1.02	
	Fibroblast	Tumor	Fibroblast	Tumor
HindIII	3.0	3.0, 8.1*	1.2	1.2
PstI	8.0	8.0, 15.5*	8.0, 12	8, 12, 15.5*
KpnI	23	23, 42.5*	6.5	6.5
EcoRV	2.7	2.7	nd	nd

FIG. 4. Map of the region of the 4.7R gene affected by the rearrangement/insertion in RB369E. The top line is a restriction map of the region surrounding the 5′ rearrangement in RB369E. Restriction enzymes are as follows: H, *Hin*dIII; M, *Mbo*I; RV, *Eco*RV; RI, *Eco*RI; P, *Pst*I; K, *Kpn*I. The lines below the map indicate the locations of the restriction enzyme fragments shown at the bottom; the normal size (in kilobases) of each fragment is indicated above the line. The insertion occurred in the region indicated by the vertical bar. Asterisks mark the junction fragments in the tabulated summary. Sizes of fragments in kilobases are shown in the tabulated summary. p7H30.7R and H3-8 contain exon sequences; p7RV1.55L and p23RI1.02 are in an intron. The *Kpn*I sites are only approximate locations. nd, Not done.

the 3.8 probe (Fig. 5c). RB355 also contained a low abundance of 4.7R transcripts (Fig. 5b). Thus, 2 of 16 RB or OS tumors which had no detectable genomic abnormality in the 4.7R gene had abnormally low levels of transcript; the other 14 tumors had amounts of 4.7R transcript on Northern blots similar to that in normal tissues.

Expression of the 4.7R transcript was observed in all normal cells tested (Fig. 5f). Most malignant tumors unassociated with mutations at the RB1 locus, i.e., neuroblastoma (IMR32), renal tumor, promyelocytic leukemia (HL-60), liver tumor, and a primitive neuroectodermal tumor cell line, Tab-1, expressed a normal-size transcript (Fig. 5a and e and 6; some data not shown).

The level of expression of 4.7R varied considerably among the various cells tested. Retinal cells from different age groups, fetal (6- to 12-weeks gestation), newborn (8 days), and adult, expressed similar levels of transcript, but two adenovirus-transformed human fetal retinal cell lines (3) contained higher levels of message (Fig. 5g and data not shown); EBV-transformed lymphocytes from normal individuals and from RB patients also expressed high levels of 4.7R, as did the rapidly growing cell lines HL60 and IMR32 (Fig. 5a and 6).

Variation in 4.7R transcript size. To obtain accurate comparisons of the sizes of different transcripts and to exclude gel artifacts, RNA from different tissues was mixed, run in the same lane of Northern blots, and probed with the 3.8 probe (Fig. 6). Two distinct bands could be resolved when the RB tumor Y79 was mixed with the other tissues, confirming the report of Lee et al. (20) that the Y79 transcript is smaller than the transcript in normal tissues or other tumors. However, we were unable to detect shortened transcripts in any other samples.

Absence of abnormal transcripts in non-malignant cells of patients with germ line mutations in the RB1 gene. The presence of 4.7R transcripts in EBV-transformed lymphocytes allowed us to search for aberrant transcripts of mutant alleles in EBV cell lines derived from individuals carrying germ line mutations in the RB1 gene. Ten EBV lines from such individuals and one EBV line from a normal individual expressed similar amounts of a 4.7-kb transcript (Fig. 5h and data not shown). No transcripts of abnormal size, as in Y79, were detected.

Anomalous results with the 0.9 probe. In studies of the genomic structure, the 0.9 probe, in addition to the expected bands, always detected at low stringency (37°C) a 14-kb *Hin*dIII fragment not present at high stringency (51°C). The four tumors with homozygous deletions, WERI-RB1, #9, RB530, and RB537, retained the 14-kb cross-hybridizing band detected with the 0.9 probe at low stringency (Fig. 2). When DNA from these tumors was digested with other enzymes and probed with 0.9, cross-hybridizing bands were also observed. The presence of these bands in all tumors otherwise deleted for the 4.7R gene and their detection only under conditions of low stringency of hybridization indicate that there is a cross-hybridizing sequence located elsewhere in the genome.

Anomalous results were also obtained in the Northern blots with this probe. In all tissues and tumors examined, including three tumors (RB530, RB537, and WERI-RB1) with homozygous deletions of the 4.7R gene, the 0.9 probe detected a 5.0-kb transcript, similar in size to the message detected with the 3.8 probe. Y79, which showed a truncated message of 4.5 kb with the 3.8 probe, showed only a 5.0-kb message with the 0.9 probe (Fig. 6). These results are consistent with the genomic studies and indicate that the 0.9

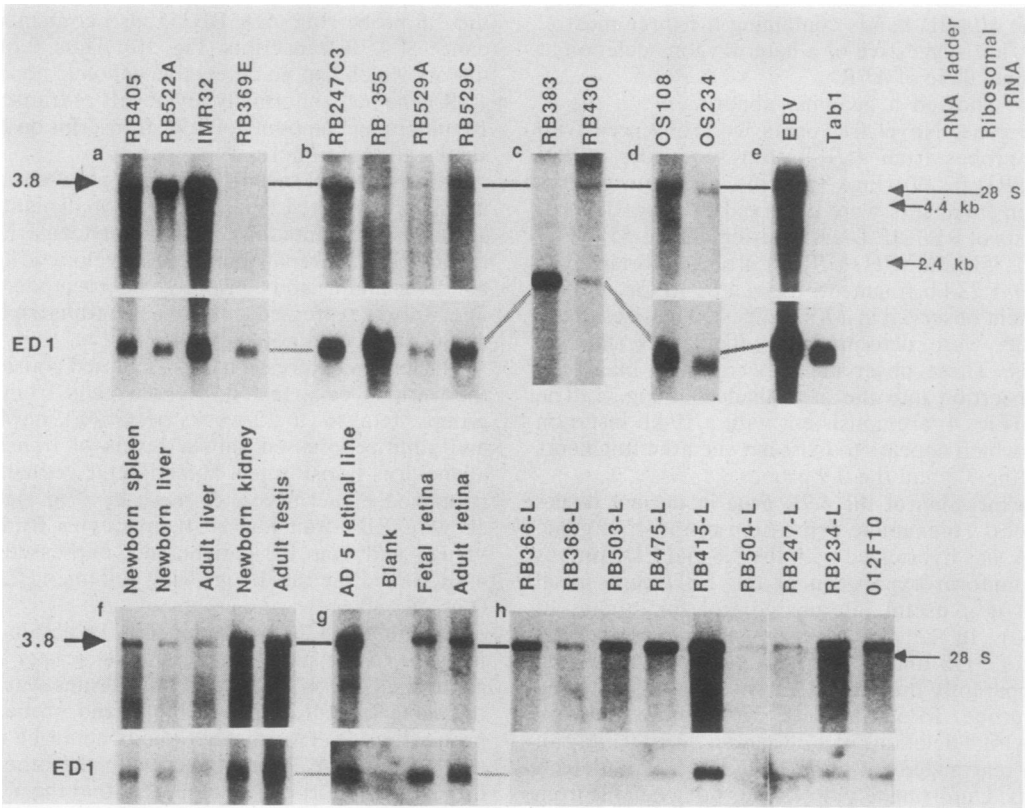


FIG. 5. Northern blot analyses of poly(A)$^+$ RNA from nine RB tumors (a, b, and c), 2 OS tumors from patients with RB (d), a primitive neuroectodermal tumor, Tab-1 (e), normal fetal, newborn, and adult tissues and an adenovirus-transformed (AD 5) fetal retinal cell line (f and g), and eight EBV cell lines from patients with germ line RB mutations and one non-RB EBV line (012F10) (h). The blots were probed with the 3.8 probe and reprobed with the ED1 subclone of the ESD gene. Two tumors were fresh surgical specimens (RB529A and RB529C); the others were tissue culture cell lines. Tab-1 expresses a normal transcript detected in a repeat Northern blot.

probe detects both the 4.7R message and a message from homologous sequences located elsewhere in the genome.

DISCUSSION

While our results with p4.7R are at variance with the previously reported results regarding gene expression, our data on the frequency of genomic rearrangements in RB and OS tumors are similar to previous data and, in general, support the identification of 47R as the RB1 gene.

Genomic rearrangement in the 4.7R gene. We considered tumors to have a structural defect in 4.7R only if an abnormality was detected in digests with more than one enzyme and was reproducible in replicate digests of different DNA preparations from that tumor. Using these criteria, we found only 4 of 34 (12%) RB and OS tumors to have rearrangements in the 4.7R gene. We confirmed the major deletions in WERI-RB1 and tumor #9 previously reported by Friend et al. (12).

Friend et al. (12) found that 30% of the 50 tumors studied with p4.7R showed homozygous deletion (6 tumors, including WERI-RB1 [tumor #41 in their report]) or rearrangement in the 4.7R gene. Lee et al. (20) confirmed the deletion in WERI-RB1 (tumor #3 in their report), showed hemizygous deletion in an RB tumor with a cytogenetic deletion involving 13q14, and found a normal genomic pattern in four RB tumors. Fung et al. (13) reported results similar to those of Friend et al. (12), with 17 of 40 (42%) tumors having genomic abnormalities. Although our frequency of confirmed genomic abnormalities (4 of 34 [12%]) is substantially lower than the overall frequency (32 of 94 [34%]) claimed by the other groups, the difference may be explained by random

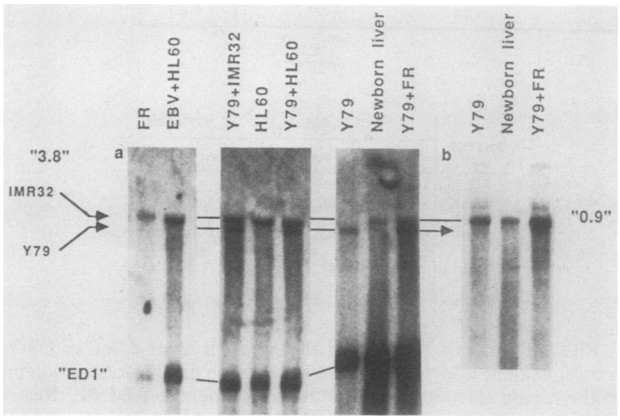


FIG. 6. Northern blot analysis of poly(A)$^+$ RNA showing the differences in transcript size between Y79 and fetal retina (FR), EBV-transformed lymphocytes and HL60, Y79 and IMR32, and Y79 and HL60. RNA samples were either run alone or mixed as indicated to ascertain size differences. (a) Blots were probed with the 3.8 probe and reprobed with the ED1 subclone of the ESD gene. (b) The blot of Y79, liver from a newborn, and mixed Y79 and fetal retina was stripped and reprobed with the 0.9 probe, showing uniform transcript size in Y79 and the other tissues.

variations in the samples tested by different laboratories or by differences in the criteria used to identify rearrangements. Half of our samples were obtained directly from surgical specimens, and the remainder were grown as cell lines in tissue culture or as xenografts in nude mice before analysis. Dividing our data along these lines does not alter the frequency of rearrangements; 2 of 19 fresh tumors and 2 of 15 cell lines showed alteration in the Southern banding pattern. Since other groups studying 4.7R (12, 13, 20) have also used both fresh and cultured tumors, it is unlikely that the source of tumor DNA accounts for the differences observed.

We detected a transcribed sequence that cross-hybridized with the 5′ end of the 4.7R gene. Since the genomic cross-hybridizing sequences are retained in the tumors completely deleted for 4.7R, it is unlikely that this gene is located in 13q14. Thus, this cross-hybridizing gene is most likely located elsewhere in the genome and may be indicative of a family of 4.7R-related genes.

Transcription of the 4.7R gene in tumors. Our detection of apparently normal 4.7R transcripts with the 3.8 probe in 12 of 17 RB tumors (including one tumor, RB369E, with both genomic and transcription abnormalities) and 2 of 2 OS tumors from RB patients differs markedly from previous reports. Some minor differences may be explained by the different techniques used to look for the mRNA. The 4.7R mRNA transcript migrates close to 28S rRNA, and we detected only a faint signal with the 3.8 probe when total cytoplasmic RNA was examined by Northern blotting. This technical difference may explain why Friend et al. (12) failed to detect any transcripts in tumors, including Y79, a tumor with a truncated transcript (Fig. 6); Lee et al. (20) first reported a truncated transcript for Y79, an observation confirmed by Fung et al. (13). Although we noted some variations in the intensities of 4.7R bands on Northern blots, the changes were not usually marked; the exceptions were for RB383 and RB355, both of which had faint bands in comparison with the band for the control transcript, ESD.

We were unable to confirm the high frequency of truncated messages reported by both Lee et al. (20) and Fung et al. (13). If the short message arose by rearrangements, one would expect to observe variations in size depending on the location of the deletions or insertions. It is also possible that the 4.7R message has alternate splicing sites leading to different-size messages in different tumors. However, this would not explain the high frequency of truncated message detected by Fung et al. (13), compared with the virtual absence of abnormal-size transcripts in 19 other RB and OS tumors reported here.

Lee et al. (22) have described a nuclear phosphoprotein with DNA-binding activity which was identified by a polyclonal antibody against a 4.7R fusion protein. They state that this protein was absent in five RB tumors in which transcripts were absent or abnormal. Although they did not examine RB tumors with normal 4.7R transcripts for the presence or absence of the protein, they did detect the protein in OS cell line, a tumor also induced by mutations in the RB1 gene (17).

Our observation of no expression in RB369E, our only tumor with demonstrable somatic rearrangements within 4.7R, is important. This unique tumor could be considered evidence that 4.7R is the RB1 gene if it can be confirmed by direct cloning that it contains an insertion in the 5′ region of the gene. Intron insertions can markedly reduce expression (18) and could create mutations leading to RB.

The message transcribed from 4.7R probably has fewer gross abnormalities that result in alterations in transcript size than were reported previously. Thus, the ultimate proof that 4.7R is or is not the RB1 gene will require unequivocal identification of somatic point mutations and small deletions within both alleles of this gene in tumors. Accordingly, using an RNase protection assay, we have analyzed RNA from tumors which appear normal on Southern and Northern blots. Point mutations or small deletions or both have been identified in four of eight tumors studied (J. Dunn et al., manuscript in preparation). Coupled with the results presented here, these data indicate that 65% of our tumors have alterations in the 4.7R gene. Since RNase protection detects approximately 50% of point mutations, these observations are consistent with all RB and OS tumors having point mutations or deletions in the RB1 gene and provide additional evidence for the identification of 4.7R as the RB1 gene.

ACKNOWLEDGMENTS

We thank T. P. Dryja for generously providing the p4.7R cDNA clone, the 3.8 and 0.9 subcloned fragments, and DNA from tumor #9; P. Gallimore for the adenovirus-transformed human fetal retina cell lines; and W. Benedict for DNA of tumor LA-RB69. We are grateful for the help of Wendy Holmes, Joan Bevilaqua, Gabrielle Fulop, and Joan Chin in culturing RB tumor cells.

This work was supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada. A.D.G. is supported by a Medical Research Council studentship. H.B. was supported by a generous donation from the Conn Smythe Foundation. J.S. is the recipient of a Steve Fonyo Studentship Award from the National Cancer Institute of Canada. B.L.G. is a Research Associate of the Ontario Cancer Foundation.

LITERATURE CITED

1. **Abramson, D. H., R. M. Ellsworth, F. D. Kitchin, and G. Tung.** 1984. Second nonocular tumors in retinoblastoma survivors. Are they radiation induced? Ophthalmol. **91**:1351–1355.
2. **Benedict, W. F., A. L. Murphree, A. Banerjee, C. A. Spina, M. C. Sparkes, and R. S. Sparkes.** 1983. Patient with 13 chromosome deletion: evidence that the retinoblastoma gene is a recessive cancer gene. Science **219**:973–975.
3. **Byrd, P., K. W. Brown, and P. H. Gallimore.** 1982. Malignant transformation of human embryo retinoblasts by cloned adenovirus 12 DNA. Nature (London) **308**:69–71.
4. **Cavenee, W. K., T. P. Dryja, R. A. Phillips, W. F. Benedict, R. Godbout, B. L. Gallie, A. L. Murphree, L. Strong, and R. L. White.** 1983. Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. Nature (London) **305**:779–784.
5. **Chirgwin, J. M., A. E Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
6. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81**:1991–1995.
7. **Draper, G. J., B. M. Sanders, and J. E. Kingston.** 1986. Second primary neoplasms in patients with retinoblastoma. Br. J. Cancer **53**:661–671.
8. **Dryja, T. P., W. Cavenee, R. White, J. M. Rapaport, R. Petersen, D. M. Albert, and G. A. Bruns.** 1984. Homozygosity of chromosome 13 in retinoblastoma. N. Engl. J. Med. **310**:550–553.
9. **Dryja, T. P., J. M. Rapaport, J. M. Joyce, and R. A. Petersen.** 1986. Molecular detection of deletions involving band q14 of chromosome 13 in retinoblastoma. Proc. Natl. Acad. Sci. USA **83**:7391–7394.
10. **Duncan, A. M. V., C. Morgan, B. L. Gallie, R. A. Phillips, and J. Squire.** 1987. Re-evaluation of the sub-localization of esterase D and its relation to the retinoblastoma locus by *in situ* hybridization. Cytogenet. Cell Genet. **44**:153–157.
11. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for

radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.

12. **Friend, S. H., R. Bernards, S. Rogelj, R. A. Weinberg, J. M. Rapaport, D. M. Albert, and T. P. Dryja.** 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature (London) **32**:643–646.
13. **Fung, Y. K., A. L. Murphree, A. Tang, J. Qian, S. H. Hinrichs, and W. F. Benedict.** 1987. Structural evidence for the authenticity of the human retinoblastoma. Science **236**:1657–1661.
14. **Gallie, B. L., D. M. Albert, J. J. Y. Wong, N. Buyukmihci, and C. A. Puliafito.** 1977. Heterotransplantation of retinoblastoma into the athymic "nude" mouse. Invest. Ophthalmol. **16**:256–269.
15. **Gallie, B. L., W. Holmes, and R. A. Phillips.** 1981. Reproducible growth in tissue culture of retinoblastoma tumor specimens. Cancer Res. **42**:301–305.
16. **Godbout, R., T. P. Dryja, J. Squire, B. L. Gallie, and R. A. Phillips.** 1983. Somatic inactivation of genes on chromosome 13 is a common event in both hereditary and nonhereditary retinoblastoma tumors. Nature (London) **304**:451–453.
17. **Hansen, M. F., A. Koufos, B. L. Gallie, R. A. Phillips, O. Fodstad, A. Brogger, T. Gedde-Dahl, and W. Cavenee.** 1985. Osteosarcoma and retinoblastoma: a shared chromosomal mechanism revealing recessive predisposition. Proc. Natl. Acad. Sci. USA **82**:6216–6220.
18. **Hawley, R. G., M. J. Shulman, H. Murialdo, D. M. Gibson, and N. Hozumi.** 1982. Mutant immunoglobulin genes have repetitive DNA elements inserted into their intervening sequences. Proc. Natl. Acad. Sci. USA **79**:7425–7429.
19. **Lee, E. Y., and W. H. Lee.** 1986. Molecular cloning of the human esterase D gene, a genetic marker of retinoblastoma. Proc. Natl. Acad. Sci. USA **83**:6337–6341.
20. **Lee, W. H., R. Bookstein, F. Hong, L. J. Young, J. Y. Shew, and E. Y. Lee.** 1987. Human retinoblastoma susceptibility gene: cloning, identification, and sequence. Science **235**:1394–1399.
21. **Lee, W. H., R. Bookstein, W. Wheatley, W. F. Benedict, and E. Y. Lee.** 1987. A null allele of esterase D is a marker for genetic events in retinoblastoma formation. Hum. Genet. **76**:33–36.
22. **Lee, W. H., J. Y. Shew, F. D. Hong, T. W. Sery, L. A. Donoso, L. J. Young, R. Bookstein, and E. Y. Lee.** 1987. The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. Nature (London) **329**:642–645.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **McFall, R. C., T. W. Sery, and M. Makadon.** 1977. Characterization of a new continuous cell line derived from a human retinoblastoma. Cancer Res. **37**:1003–1010.
25. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13**:7207–7221.
26. **Reid, T. W., D. M. Albert, A. S. Rabson, P. Russell, J. Craft, E. W. Chu, T. S. Tralka, and J. L. Wilcox.** 1974. Characteristics of an established cell line of retinoblastoma. J. Natl. Cancer Inst. **53**:347–360.
27. **Sakai, K., N. Kanda, Y. Shiloh, T. Donlon, R. Schreck, J. Shipley, T. Dryja, E. Chaum, R. S. Chaganti, and S. Latt.** 1985. Molecular and cytologic analysis of DNA amplification in retinoblastoma. Cancer Genet. Cytogenet. **17**:95–112.
28. **Schwab, M., H. E. Varmus, J. M. Bishop, K. H. Grzeschik, S. L. Naylor, A. Y. Sakaguchi, G. Brodeur, and J. Trent.** 1984. Chromosome localization in normal human cells and neuroblastomas of a gene related to c-*myc*. Nature (London) **308**:288–291.
29. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
30. **Squire, J., B. L. Gallie, and R. A. Phillips.** 1985. A detailed analysis of chromosomal changes in heritable and non-heritable retinoblastoma. Hum. Genet. **70**:291–301.
31. **Squire, J. A., T. P. Dryja, J. Dunn, A. D. Goddard, T. Hofmann, M. Musarella, H. F. Willard, A. J. Becker, B. L. Gallie, and R. A. Phillips.** 1986. Cloning of the esterase D gene: a polymorphic gene probe closely linked to the retinoblastoma locus on chromosome 13. Proc. Natl. Acad. Sci. USA **83**:6573–6577.
32. **Squire, J. A., A. D. Goddard, M. Canton, A. Becker, R. A. Phillips, and B. L. Gallie.** 1986. Tumor induction by the retinoblastoma mutation is independent of N-*myc* gene expression. Nature (London) **322**:555–557.
33. **Vorbrodt, A., P. Meo, and G. Rovera.** 1979. Regulation of acid phosphatase activity in human promyelocytic leukemic cells induced to differentiate in culture. J. Cell Biol. **83**:300–307.
34. **Winger, L., C. Winger, P. Shastry, A. Russell, and M. Longnecker.** 1983. Efficient generation in vitro, from human peripheral blood cells, of monoclonal Epstein-Barr virus transformants producing specific antibody to a variety of antigens without prior deliberate immunization. Proc. Natl. Acad. Sci. USA **80**:4484–4488.