Studies on the Human Retinoblastoma Susceptibility Gene

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The retinoblastoma susceptibility (RB) gene is unique among other cloned cancer
genes because its causal role in a human cancer, retinoblastoma, was established
by classical genetic methods before its isolation. Earlier hypotheses and experi-
mental data suggested that inactivation of a gene in chromosome band 13q14
resulted in retinoblastoma formation. A gene in this region was identified as the
RB gene on the basis of mutations found specifically in retinoblastoma tumors;
however, its proposed biological activity in suppressing neoplasia has yet to be
demonstrated. The RB gene product was identified as a nuclear phosphoprotein of
110 kD associated with DNA binding activity, suggesting that the RB protein may
regulate other genes. Probes for the RB gene and gene product will be useful for
genetic diagnosis of retinoblastoma susceptibility in affected families; for direct
detection of mutant RB alleles; and, potentially, for genetic diagnosis of suscepti-
bility to osteosarcoma and other tumors tentatively linked to RB-gene dysfunction.
Continued study of the RB gene should yield further insight into mechanisms of
oncogenesis, development, and gene regulation.

GENETICS OF RETINOBLASTOMA

It is an undisputed observation that the cancer phenotype necessarily involves
genetic alterations within tumor cells [1–3]. Some of these alterations may occur in
somatic cells during the life of an individual; other mutations might be inherited from
a parental germline. The latter type of inheritance would explain cases of familial
cancer and inherited cancer predisposition [4,5]. The clearest example of a heritable
cancer predisposition is that afforded by retinoblastoma, a highly malignant but
readily treatable cancer of the retina that occurs in young children. Its incidence is
about 1 in 20,000 live births, and it is the most common intraocular tumor of the
pediatric age group [6].

Two forms of retinoblastoma are distinguished on a genetic basis [7]. The hereditary form (comprising 40% of all cases) is an autosomal dominant cancer susceptibility trait: each offspring of a carrier parent has a 50% chance of inheriting the trait, and 90% of carriers will develop retinoblastoma [8,9]. Multiple or bilateral retinal tumors are indicative of, and typical for, hereditary retinoblastoma [6]. Furthermore, carriers are at high risk of developing additional primary neoplasms later in life; these second cancers are of otherwise unusual types, such as osteosarcoma or fibrosarcoma, and are often fatal [10,11]. In contrast, patients with nonhereditary retinoblastoma have single, unilateral retinal tumors and no increased risk of second cancers [6]. However, about 15% of patients with unilateral retinoblastoma actually have the hereditary form [9]. Because of its clear-cut heritability, retinoblastoma has been a prototypic model for the study of genetic determination in cancer [5]. Other cancers with known familial occurrence include nephroblastoma (Wilm's tumor) [5], neuroblastoma [5], osteosarcoma [12], renal cell carcinoma [13], melanoma [14], and breast cancer [4].

Using a statistical analysis of clinical data as a basis, Knudson inferred that retinoblastoma could result from as few as two "hits," or mutational events [15]. Comings [16] hypothesized that two hits served to inactivate both alleles of a single gene (RB) that essentially functioned to suppress retinoblastoma formation. An individual inheriting a mutant RB allele in all somatic cells would be predisposed to getting retinoblastoma by an additional mutation of the other RB allele in one precursor cell (retinoblast). In sporadic cases, both RB alleles would have to be inactivated by two independent somatic mutations in a single retinoblast. This model could explain both the earlier onset and multiplicity of tumors in predisposed individuals. However, the validity of this hypothesis remained to be demonstrated at the molecular level.

CHROMOSOME REGION 13q14 INVOLVEMENT IN RETINOBLASTOMA

Karyotypic examination of somatic cells (fibroblasts) from patients with hereditary retinoblastoma disclosed a minor subset of cases containing visible deletions of the long arm of chromosome 13 [17,18]. Similar deletions were also identified in retinoblastoma tumor cells [19]. Studies of a large retinoblastoma pedigree showed that normal individuals carried a balanced translocation involving 13q14, whereas those with retinoblastoma had only one 13q14 region [20]. Band 13q14 was common among all deletions and presumably contained a gene (RB) that determines susceptibility to hereditary retinoblastoma; these deletions also removed one allele of the gene for a polymorphic marker enzyme, esterase D [21]. Close linkage of these two loci was confirmed by studies of retinoblastoma pedigrees [22]. Godbout et al. [23] showed a loss of one esterase D allele in retinoblastomas from 4 out of 6 patients heterozygous for esterase D, and Cavenee et al. [24] demonstrated specific loss of heterozygous chromosome 13 markers in retinoblastomas, compared with somatic cells from the same patients. These reports indicated that partial or complete loss of one chromosome 13 was a common event during retinoblastoma genesis. In light of Knudson's two-hit hypothesis, loss of this chromosome was interpreted as the "second hit" that revealed mutations of the other RB allele ("first hit"). Although the nature of this presumed mutation was unknown, if it served to inactivate one RB allele, then tumor formation would be associated with complete dysfunction of the RB gene.

Benedict et al. described a case of bilateral retinoblastoma in which both RB alleles were inferred to be absent from the tumor [25]. Recently an assumption made in this case has been disproved [26], namely, that absence of esterase D activity implied loss of both esterase D and RB genes. However, Dryja et al. [27] found two retinoblastomas with small homozygous deletions detected by probe H3-8 but not by other probes mapping to 13q14, providing the first molecular evidence for complete absence of gene(s) in this region. These studies are consistent with the notions that the mutant RB allele is "recessive" to its normal counterpart within a cell and that the latter functions to prevent tumor formation in the retina. Tumorigenic activity in the absence of gene function distinguishes this presumed "cancer suppressor gene" from classical oncogenes, which require the presence of (altered) gene products for tumor formation [28].

MOLECULAR CLONING OF GENES FROM CHROMOSOME REGION 13q14

Since nothing was known a priori about the RB gene product, candidate genes were to be identified solely on the basis of appropriate chromosomal location and presumed "recessive" behavior, as described earlier; that is, an intact RB gene should be expressed in normal retinal tissue but not in retinoblastomas. "Reverse genetic" cloning strategies require a collection of one or more DNA probes from the region of interest. These may consist of probes for other known genes or of anonymous DNA probes isolated at random by a number of techniques. Before attempting to clone the RB gene, several laboratories made major efforts to obtain probes for region 13q14. The polymorphic marker enzyme esterase D was mapped to 13q14 and is closely linked to the RB gene, with no known recombinants [8]. By generating specific antisera and partially sequencing the protein, we and others have identified esterase D cDNA fragments [29–31]. Also available were anonymous DNA probes mapping to 13q14, such as H3-8, H2-42 [32], and 7D2 [33] that were isolated by random selection from chromosome 13–specific libraries.

We first initiated bidirectional chromosome walking from the esterase D gene to create contigs (regions of overlapping genomic clones) of progessively larger size. At 20-kb intervals in walking regions, unique sequences were identified that were used as probes to isolate cDNA clones from fetal retina and placenta libraries. By alternately screening genomic and cDNA libraries, we established a contig covering 120 kilobases around the esterase D gene. Two cDNA clones, called SD-1 and SD-2, were isolated with probes 5' to the esterase D gene. Chromosome walking 3' to the esterase D gene was hampered by a 20-kb region containing highly repetitive sequences. A second bidirectional chromosome walk extending over 30 kb was started from probe H3-8, which was found to be homozygously deleted in two retinoblastoma tumors [27]. A unique DNA fragment in this region identified two overlapping cDNA clones of 1.6 kb (RB-1) and 0.9 kb (RB-2) in human cDNA libraries (Fig. 1).

IDENTIFICATION OF THE RB GENE

We expected that candidate RB genes would be expressed in fetal retinal cells but not in retinoblastoma cells. Therefore, cDNA clones isolated above were used as probes in RNA blotting analysis to detect mRNA transcripts in fetal retina, normal human placenta, and cultured cells from six retinoblastomas, three neuroblastomas,
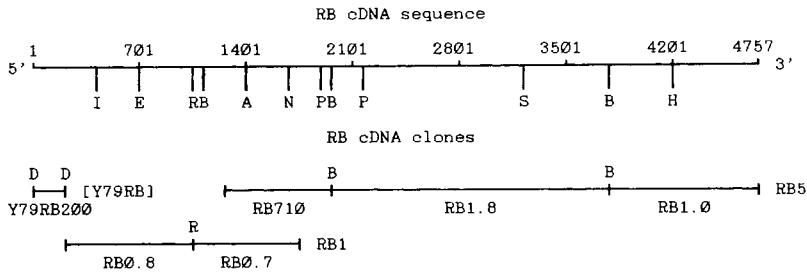
RB cDNA sequence

RB cDNA clones

Fig. 1. cDNA clones of the RB gene and alignment to the full-length sequence. Restriction sites are indicated as follows: A, *Acc* I; B, *Bgl* II; D, *Dde* I; E, *Eco*R V; H, *Hin*d III; I, *Hpa* I; N, *Nco* I; P, *Pst* I; R, *Eco*R I; S, *Stu* I.

TABLE I. mRNA Expression of Genes in Region 13q14

Cells	Probes			
	Est[a]	SD-1	SD-2	RB-1
Placenta	+	− (+)	−	+
Fetal retina	+	−	− (+)	+
Retinoblastoma				
1	+	−	−	+/alt
2	+	−	−	+/alt
3	+	−	−	−
4	+	−	−	±/alt
5	+	−	−	+/alt
6	+	−	−	−
Neuroblastoma				
1	+	−	−	+
2	+	−	−	+
3	+	−	−	+
Medulloblastoma				
1	+	ND	ND	+
2	+	ND	ND	+

[a]Abbreviations: Est, esterase D; +, detectable mRNA expression; −, no detectable expression; (+), expression expected; ND, not done; alt, altered mRNA size.

and two medulloblastomas. Table I summarizes RNA hybridization results. Esterase D transcripts were detected in all tumor and tissue samples (Fig. 2, bottom panel), which is consistent with the known "constitutive" expression of esterase D [29]. All esterase D mRNA transcripts had identical size (1.4 kb). Neither SD-1 nor SD-2 seemed promising as candidate RB genes, because transcripts hybridizing to these clones were not detected in retina and placenta mRNA samples or in any retinoblastomas (Table I).

Clone RB-1 detected a 4.7-kb mRNA transcript in fetal retina and placenta (Fig. 2). Three retinoblastomas (lanes 1, 2, 5) demonstrated abnormal mRNA transcripts measuring approximately 4.0 kb (Fig. 2A). In two retinoblastomas (lanes 3, 6), mRNA transcripts were not observed, and faint bands of about 3.8 and 4.5 kb were visible in lane 4 only after prolonged exposure. Three neuroblastomas and two medulloblastomas displayed identical transcripts of 4.7 kb, equivalent to those in normal tissues (Fig. 2B). Alterations in gene expression therefore were found in 6 out of 6 retinoblastomas, but not in two normal tissues and two other related human

A   **B**

1 2 3 4 5 6 7   1 2 3 4 5 6

(Kb)   (Kb)

4.6—   —4.6
4.0—

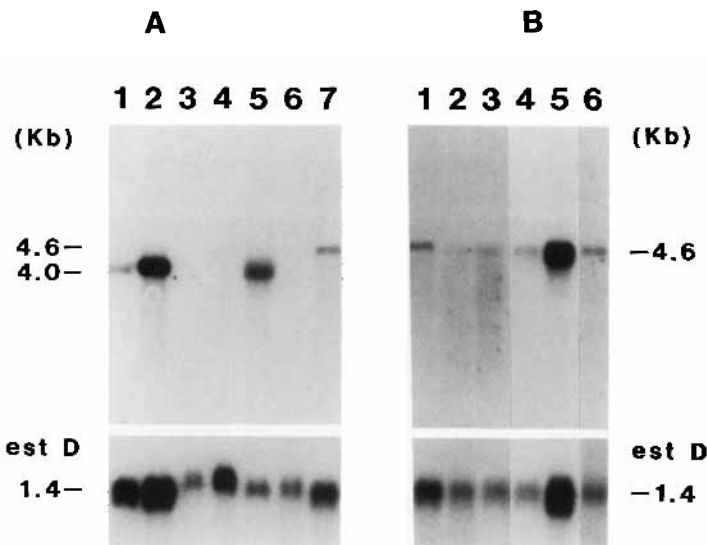est D   est D
1.4—   —1.4

Fig. 2.   RNA blot analysis of RB gene transcripts in tumors and normal tissues. Two to five micrograms of polyadenylated RNA prepared from retinoblastoma cell lines Y79, RB355, WERI-1, WERI-24, and WERI-27 (**lanes A1–5**), short-term cultured cells from a primary retinoblastoma tumor (**lane A6**), fetal retina (**lane A7**), neuroblastoma cell lines (**lanes B1–3**), a medulloblastoma cell line and a fresh tumor (**lanes B4, 5**), and human placenta ((**lane B6**) were electrophoresed in 1% formaldehyde-agarose gels and transferred to nitrocellulose filters with 20 × SSC. Filters were hybridized with ^{32}P-labeled RB-1 DNA (**top panel**) in 50% formamide, 6 × SSC, 5 × Denhardt's solution, 0.1 % SDS, 100 ug/ml denatured salmon sperm DNA, 10 mM phosphate buffer (pH 7.0) at 42°C for 24 h. Washing followed in 2 × SSC, 0.1% SDS, at RT for 20 min once and in 0.1 × SSC, 0.1% SDS, at 65°C for 30 min twice. Filters were autoradiographed on Kodak XAR-5 film at −70°C for 3 days with an intensifying screen. Tumors initially showing no signal were retested by overloading lanes with 10 ug of polyadeny-lated RNA and autoradiographing for up to 10 days. After this procedure, tumor 4 demonstrated an additional faint band of 3.8 kb (data not shown). Filters were then rehybridized with ^{32}P-labeled EL-22 DNA, as described above, and exposed for 3 days (**bottom panel**). The apparent slight variation in mobility of esterase D mRNA transcripts reflects the required overloading.

tumors of neurectodermal origin. This finding constituted the strongest evidence that RB-1 represented part of the putative RB gene [34]. Friend et al. [35] first reported a cDNA fragment isolated by chromosome walking from H3-8 [27]. Both Friend et al. and Fung et al. [36] detected a gene with properties similar to those described above: ubiquitous expression in normal tissues but absent or altered transcription in retinoblastomas.

Partial or complete deletions of the RB gene may be detected by absent or altered bands in genomic Southern blotting analysis. Homozygous internal deletions have been considered strong evidence in favor of correct identification of the RB gene. Both Friend et al. and Fung et al. reported a few cases (including one osteosarcoma) with homozygous internal deletions of the RB gene. However, the size and complexity of the RB gene (see below) and the relative insensitivity of Southern blotting make accurate analysis of genomic rearragements somewhat problematic. Caution is warranted in making inferences of deletions based solely on decreased band intensities; detection of deletion junction fragments is more reliable. For exam-ple, osteosarcoma OHS, which was reported to have a homozygous internal deletion [36], in fact demonstrated both altered and normal bands and therefore heterozygous

for its deletion (unpublished data). Conversely, despite two reports of grossly normal DNA structure [34,36], retinoblastoma cell line Y79 has a heterozygous internal deletion (see below). Whereas a majority of reported tumors had no apparent genomic alterations detectable by Southern blotting with cDNA probes [34–36], DNA rearrangements in one or both alleles may be more frequent than currently suspected.

COMPLETE cDNA SEQUENCE AND GENOMIC ORGANIZATION OF THE RB GENE

Additional RB cDNA clones were obtained by rescreening several cDNA libraries with RB-1 as probe (Fig. 1). Clone RB-5 (3.5-kb insert) overlapped RB-1 by restriction and sequence analysis. Another RB cDNA clone was isolated that extended an additional 242 base pairs beyond the 5' end of RB-1 [37]. Together these clones defined a cDNA sequence of 4,757 nucleotides [38] with a long reading frame open from the 5' end. We favor as the true initiation codon the first in-frame Met (nucleotide 139) instead of the second (nucleotide 475) for the following reasons: 1) MW of the predicted protein translated from the first Met was closer to the actual MW of the RB gene product in vivo (see below); 2) in vitro translation yielded full-size product only when the first 200 nucleotides were present (unpublished data). With the first methionine, the predicted RB protein had 928 amino acids and a molecular weight of 110 kD. A CG-rich sequence (88%, nucleotides 166–225) encoded an unusual stretch of amino acids (AATAAAAAAEPPAPPPPPPP, residues 10–29). We speculate that this region of DNA or protein is associated with some special function. Other features of the predicted amino acid sequence included two atypical potential metal-binding domains similar to those found in nucleic acid-binding proteins [39]. However, the RB protein had no close relatives in current protein sequence databases.

Genomic clones of the RB gene were obtained by screening genomic libraries with cDNA probes. More than two dozen nonredundant, overlapping phage clones assorted into three overlapping groups (contigs) according to shared restriction fragments and shared exons [40]. The total span of genomic clones exceeded 150 kb. By Southern blot analysis, the first two contigs were separated by only 1.5 kb (unpublished data). Comparison with mapping by another group (T. Dryja, personal communication) has shown that our other internal gap measures about 50 kb; therefore total gene size is approximately 200 kb. Exons were initially identified as minimal-length EcoRI and/or HindIII restriction fragments containing sequences hybridizing to RB cDNA clones. About 50 oligonucleotides have been synthesized and located in the exon map by hybridizing to DNA blots of genomic clones. These oligonucleotides were used as primers to define additional exon/intron junctions by sequencing genomic clones. Based on this detailed characterization, the RB gene contains 27 exons (Huang et al., unpublished). Additional exons were excluded from the two gap regions, which consequently contained only intron sequences.

The 5' untranslated portion and first methionine were found in a single exon (exon 1) of the genomic map. The coincidence of cDNA length (4,757 bp) and mRNA size (4.7 kb), as well as primer extension studies (unpublished), indicates that our cDNA sequence was essentially complete and that the first exon was correctly located. Restriction and sequence analysis demonstrated that the last exon was 1.9 kb long and included the translation stop codon (nucleotides 2924–2926).

THE RB GENE PRODUCT

Using the hypothetical protein sequence data from our initial report [34], we constructed a recombinant plasmid, pATH-0.7, that expressed a TrypE-RB fusion protein in *E. coli*. Rabbits were immunized with this fusion protein, and the resulting antiserum was purified on affinity columns containing TrypE or fusion proteins. The purified polyclonal antibody immunoprecipitated a phosphoprotein of about 110 kD in normal cells that was specifically absent in cultured cells from five out of five retinoblastomas. On this basis, we identified the detected protein as the RB gene product, pp110^{RB} (Fig. 3) [38]. This protein was located in the cell nucleus and was associated with DNA binding activity, supporting its proposed role in regulating other genes. The fusion protein antiserum serves as a specific probe for the RB protein. Several other polyclonal antibodies against RB peptides have since been produced that also precipitate pp110^{RB} (unpublished).

MECHANISMS OF RB GENE INACTIVATION

Common mutations are clearly associated with such recessive genetic diseases as sickle cell anemia and phenylketonuria, which are maintained by selective pressure or have spread by a founder effect [41–44]. These kinds of mutations were not expected in the RB gene, because most RB mutations arise de novo [9]. On the other hand, genomic rearrangements in the LDL genes (in familial hypertriglyceridemias) and hemoglobin genes (in thalassemias), in many cases occur at *Alu* repetitive
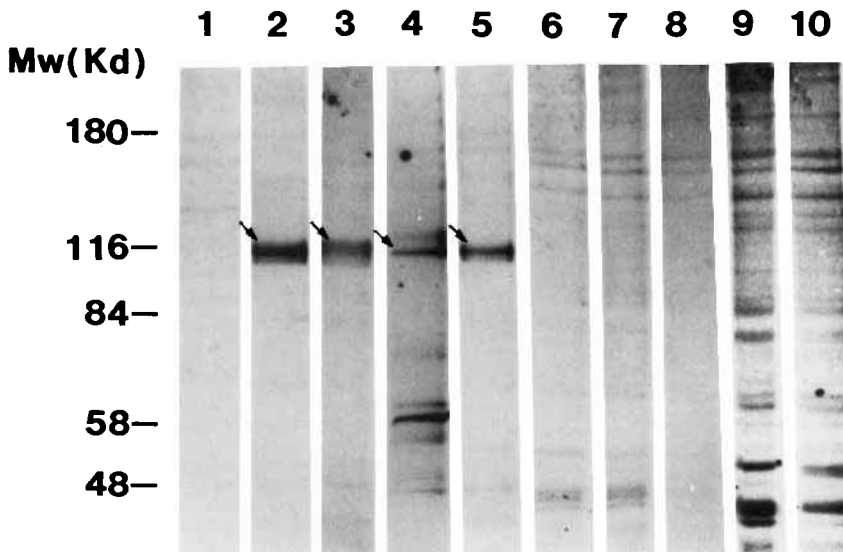

Fig. 3. Identification of RB proteins by immunoprecipitation with rabbit anti-RB IgG. Human cells such as neuroblastoma LAN-1 (**lanes 1, 2**), Alexander hepatoma (**lane 3**), osteosarcoma U20S (**lane 4**), normal fibroblasts (**lane 5**), and five retinoblastomas (**lanes 6–10**) were labeled with ^{35}S-methionine and immunoprecipitated with preimmune rabbit IgG (**lane 1**) or rabbit anti-RB IgG (**lanes 2–10**). The immunoprecipitates were analysed by 7.5% SDS-polyacrylamide gel electrophoresis and autoradiographed. The specific proteins (**arrows**) were not present among nonspecific background bands in lanes 6–10, despite prolonged exposure. **Markers (left)** show Mr in thousands.

sequences, presumably due to unequal crossing over during meiotic or mitotic recombination [45–47]. Commonly deleted regions have also been observed within the Duchenne muscular dystrophy gene [48].

To understand mechanisms of RB gene inactivation, we have examined in detail mutations affecting the RB gene in retinoblastoma cell line Y79 [49]. This established cell line expressed a shortened RB mRNA transcript (Fig. 2A, lane 1), but RB DNA appeared grossly normal [34,36]. Y79 genomic DNA was further analyzed by Southern blotting with a unique sequence probe in intron 1 [40]. Unlike cDNA probes, this probe detected novel restriction fragments with multiple endonucleases in Y79 DNA, compared with normal DNA, suggesting heterozygous genomic rearrangement. A genomic library constructed from DNA of Y79 yielded two clones, Y2.3 and Y7.3, defining a deletion of about 50 kb that removed exons 2–6 from one RB allele. Heterozygosity was confirmed because other Y79 clones contained these exons in their proper positions. In addition, two other retinoblastomas that expressed shortened RB transcripts were also found to have similar heterozygous deletions by Southern blotting with the intron-1 probe. The same probe also detected genomic rearrangements in fibroblasts from two hereditary retinoblastoma patients. Therefore, the region around intron 1 appears to be commonly involved in rearrangements that inactivate the RB gene. Because normal-sized RB mRNA and RB protein were absent from Y79 cells, the other RB allele also was inactivated by different mechanisms than the first; a mutation of the promoter is considered most plausible. Probes for detecting these presumptive promoter mutations as well as those in other common mutational sites may have useful clinical application.

In a complementary approach, a cDNA library was constructed with mRNA from Y79, and clones were isolated by screening with portions of the normal RB cDNA [37]. Sequence analysis showed that clones from the 5' portion of the transcript lacked 470 nucleotides corresponding to the aforementioned deletion of exons 2–6. Possible mechanisms involved in generating the RB gene deletion in Y79 were further explored by sequencing the cloned deletion junction as well as corresponding regions in the normal gene [37]. Sequences of the deletion endpoints demonstrated no apparent homology to each other or to the *Alu* family; therefore, the deletion cannot be explained by recombination between homologous sequences. A computer-assisted search of several GENBANK databases was also uninformative about the nature of the deletion endpoints. One proposed site-specific mechanism involves intrinsic organization of chromatin loops by chromosome scaffolding proteins [50]: Darras and Franke [51] and Vanin et al. [52] have suggested a role for topoisomerase II (which may act at points of scaffolding attachment) in generating certain gene deletions.

ONCOGENIC OR ANTIONCOGENIC ACTIVITY OF THE RB GENE

Genetic identification of the RB gene as described earlier must be further confirmed by assays for its expected biological activity. Such assays will be similar to, but the converse of, transfection experiments with classical oncogenes: Can the presence of normal RB expression prevent or suppress retinoblastoma formation? Or can the absence of expression (alone or in combination with other factors) induce retinoblastoma? For example, retinoblastoma cells can be transfected with the normal RB gene and tested for loss of malignant phenotype, either by morphologic changes in culture or by tumorigenicity in nude mice. In theory, cells may fail to "revert"

because, by the concept of multistep carcinogenesis, altering the initiating factor does not necessarily reverse the process [2,28]. However, it was recently reported [53] that introduction of human chromosome 11 into a Wilm's tumor cell line resulted in suppression of tumorigenicity in nude mice; at the same time, these cells retained a transformed phenotype in culture (chromosome 11 carries the proposed suppressor gene for Wilm's tumor). Because an entire chromosome was transferred, this activity cannot be ascribed with certainty to a single gene. An urgent question to be resolved is whether the RB gene alone can suppress tumorigenicity of certain transformed cells.

On the other hand, the RB gene product in normal cells can be inactivated by one of several possible methods and the resultant cells observed for the transformed phenotype. In yeast, specific gene conversion has been successfully achieved by homologous recombinant integration of known genetic elements [54]: for example, cytoskeletal genes have been disrupted for functional analysis of their products [55,56]. This approach is referred to as gene targeting [54]. An elegant experiment in the eukaryotic unicellular organism *Dictyostelium discoideum* was recently reported in which a transfected plasmid was integrated into the myosin heavy chain gene by homologous recombination, resulting in defective cytokinesis and multinucleation [57]. Gene targeting in diploid cells has also been reported, but it occurs at extremely low frequency [58]. Fibroblasts derived from retinoblastoma patients with visible chromosome 13q14 deletions are available [30]; because these cells contain only a single copy of the RB gene, they may be ideal target cells for this experiment.

An alternative method for specifically inactivating a gene is to block it at the mRNA level by so-called "antisense RNA inhibition" [59]. This technique was first carried out successfully in mouse cells with a thymidine kinase gene [60]. Double-stranded thymidine kinase RNA was found only in the nuclei of such cells and appeared to be unable to exit to cytoplasm. Antisense RNA has also been used to inhibit the expression of the discoidin genes of *Dictyostelium* [61] and the *hsp* 26 and *Krupple* genes of Drosophila [62,63]. Recently, the function of myosin in *Dictyostelium* cells has been studied by inhibiting expression of the myosin heavy chain gene with antisense RNA [64]. This approach was successful in inactivating a specific gene in diploid cells. Inactivation of the RB gene either by gene targeting or antisense RNA inhibition are important experiments to delineate the oncogenic potential of the RB gene.

THE RB GENE IN OTHER CANCERS

By analogy to retinoblastoma, a class of "cancer suppressor genes" [28] has been postulated to explain other types of inherited cancers and tumor-specific chromosomal deletions [65]. Because retinoblastomas and osteosarcomas often occurred in the same patients, the latter tumors were also examined with polymorphic markers on chromosome 13. Five out of seven osteosarcoma tumors, from patients both with and without retinoblastoma history, showed selective loss of heterozygous chromosome 13 markers; no other chromosomes showed consistent loss [66]. This finding suggested that retinoblastoma and osteosarcoma occurred by similar genetic mechanisms and, further, that both might involve the same gene, RB. Even more suprisingly, four out of ten breast cancer cases (of several histologic types) showed similar, specific loss of chromosome 13 heterozygosity [67], again indirectly suggesting RB

involvement. Alternatively, a different cancer suppressor gene on chromosome 13 might be present. Loss of heterozygosity has been found at chromosome 11p in Wilm's tumor [68–70] (consistent with known deletions), tumors of the Beckwith-Wiedemann syndrome [71], transitional cell carcinoma of the bladder [72], and, again, breast cancer [73]. The latter finding may indicate cooperation between different cancer suppressor genes, or genetic heterogeneity. Similarly, other suppressor loci have been implicated in neuroblastoma [74], small-cell lung carcinoma [75], renal-cell carcinoma [76], acoustic neuroma [77], and colorectal carcinomas [78,79].

The RB gene was initially identified on the basis of altered RB gene expression in retinoblastoma tumors, compared with that of normal retina, placenta, and nonretinoblastoma tumors. We have since examined many additional tumors and neoplastic

TABLE II. Summary of Cell Lines and Tumors Tested for RB Gene Mutation*

Cell line or tumor	RB DNA	RB mRNA	ppRB[110]
Retinoblastoma			
Cell lines			
Y79	+/del	+/alt	−
RB355	+/del	+/alt	−
WERI-1	−	−	−
WERI-24	+/hem	−	−
WERI-27	+/del	+/alt	−
6	+	−	ND
Tumors			
SDRB1	+	ND	ND
SDRB2	+	ND	ND
Cultured fibroblasts from RB patients			
SDRB1	+	ND	+
SDRB2	+	ND	+
S362	+	ND	+
GM1142	+	ND	ND
5	+	ND	+
6	+/alt	ND	ND
Osteosarcoma			
Cell lines			
G292	+	+	+/alt
TE85	+	+	+
143B	+	+	+
SAOS2	+/del	+/alt	−
U2OS	+	±	+
MG63	+	+	+
OHS	+/del	+/alt	−
KPD	ND	ND	+
Tumors			
1	+	+	ND
2	+	+	ND
3	+	+	ND
4	+	+	ND
Neuroblastoma			
Cell lines			
LAN-1	+	+	+
LAN-5	+	+	+

(continued)

TABLE II. Summary of Cell Lines and Tumors Tested for RB Gene Mutation* (continued)

Cell line or tumor	RB DNA	RB mRNA	ppRB[110]
Medulloblastoma			
Cell line			
TE671	+	+	ND
Tumor			
1	+	+	ND
Synovial sarcoma			
Cell line			
GHM	+	+	−
Tumor			
1	+/alt	+/alt	ND
Unclassified sarcoma			
Cell line			
MHM	+	+	+
Glioblastoma			
Cell lines			
1	+/alt	ND	ND
2	+	ND	ND
3	+	ND	ND
4	+	ND	ND
5	+	ND	ND
Mammary tumors			
Cell lines			
1 MDA–MB436	+/alt	+/alt	−
2 MDA–MB438	+/del	−	−
3 MDA–MB–134 VI	ND	ND	+
4 MDA–MB–157	ND	ND	+
5 MDA–MB–361	ND	ND	+
6 MDA–MB–175–VII	ND	ND	+
7 BT–483	ND	ND	+
8 MCF-7	ND	ND	+
9 MDA–MB–435S	ND	ND	+

*Genomic DNA and mRNA were analyzed by Southern blotting analysis with cDNA probes; certain cases (e.g., retinoblastoma Y79) were further characterized as described in the text. In all samples with total absence of hybridization, the adequacy of sample loading was confirmed by rehybridizing with esterase D cDNA as probe. The presence or absence of protein was determined by immunoprecipitation with an antibody against the RB fusion protein. Loaded protein was biochemically quantified. Abbreviations: +, normal; −, absent; +/alt, altered size of mRNA or rearrangement of DNA; +/del, DNA deletion; +/hem, hemizygous by gene dosage; ND, not determined.

cell lines and have found several nonretinoblastoma tumors or cell lines with either DNA rearrangements or altered RB mRNA. These results are collected in Table II. For example, of six osteosarcoma cell lines tested, two contained DNA deletions and expressed shortened mRNA transcripts of the RB gene (Table II). DNA and mRNA from four fresh-frozen osteosarcoma tumors appeared normal on analysis with cDNA probes; however, one fresh synovial sarcoma tumor demonstrated a lengthened mRNA in addition to one of normal size [80]. Partial DNA deletions and absent or altered mRNA have also been found in two breast cancer cell lines [84]. These preliminary results suggest a role for RB gene mutation in the genesis of cancers other than retinoblastoma. However, for each tumor type, mutations have been

detected in only a relatively small fraction of cases. There are two possible explanations: 1) there is genetic heterogeneity among these tumors, so that only a fraction of each tumor type involves the RB gene, and/or 2) we are not detecting most mutations with our cDNA probes. A combination of these two explanations may be closest to the truth.

POTENTIAL FOR CLINICAL APPLICATIONS

Restriction fragment length polymorphisms in or near the RB gene were anticipated to be very useful for genetic counseling in families segregating for mutant RB alleles. For example, we have found an RFLP for endonuclease *Bam*HI in intron 1 that was heterozygous in about 50% of individuals [40]. In a study by Wiggs et al. [81], at least one in five RFLPs within the RB gene was heterozygous and informative in 19 out of 20 retinoblastoma kindreds. RFLPs cosegregated with retinoblastoma susceptibility in all but one family, the latter having a critical proband with an uncertain clinical diagnosis. Despite this, the calculated LOD score was highly significant. Because the esterase D gene is closely linked to RB (no known recombinants), a polymorphic *Apal* site in esterase D is also of predictive value [31,82]. These probes can be used in a panel of RFLPs for genetic diagnosis in retinoblastoma and Wilson's disease [83] families.

Direct detection of RB gene mutations would have great clinical utility for the following reasons: 1) sporadic unilateral hereditary and nonhereditary cases could be distinguished by examining patients' fibroblasts, allowing accurate assessment of risk for second primary cancers and for transmission to offspring, and 2) genetic diagnosis would be possible without informative RFLPs or without examining other family members. As we described earlier, the intron-1 probe may be useful for this purpose, depending on what fraction of mutant RB genes have rearrangements in this region. Other common sites of mutation in the RB gene might be identified, and probes could be designed specifically for their detection. It is possible that the RB gene promoter may be one such common site.

Antibodies to the RB protein may have diagnostic and/or prognostic application in clinical medicine. For example, mutations in the RB gene could be inferred by absence of immunoperoxidase staining of tumor sections, with nonneoplastic stroma providing an internal positive control. RB antibodies might be used to resolve ambiguities in tissue diagnosis of bone or soft-tissue neoplasms; perhaps breast cancers could be usefully subclassified on the basis of RB gene involvement.

Finally, if inactivation of the RB gene is the primary cause of retinoblastoma and other cancers, then restoration of normal RB gene activity by gene transfer is a novel approach for future cancer therapy.

CONCLUSIONS AND PERSPECTIVE

Isolation of the RB gene is an important milestone in cancer research. It is the first cloned human gene conferring a heritable predisposition to cancer; it is also the first cloned "cancer suppressor" gene. Probes for the RB gene and gene product will be useful for genetic diagnosis of retinoblastoma susceptibility in affected families; for direct detection of mutant RB alleles; and, potentially, for genetic diagnosis of susceptility to osteosarcoma and other tumors tentatively linked to RB gene dysfunc-

tion. Continued study of the RB gene should yield further insight into mechanisms of oncogenesis, development, and gene regulation.

ACKNOWLEDGMENTS

REFERENCES

1. Klein G: Science 238:1539–1545, 1987.
2. Bishop JM: Science 235:305–311, 1987.
3. Klein G, Klein E: Nature (London) 315:190–195, 1985.
4. Knudson AG: Adv Cancer Res 17:317–352, 1973.
5. Knudson AG: Cancer 35:1022–1026, 1975.
6. Shields JA: "Diagnosis and Management of Intraocular Tumor." St. Louis: CV Mosby, 1983, pp 437–439.
7. Francois J, Matton MT, DeBie S, Tanaka Y, Vandenbulcke D: Opthamologica 170:405–425, 1976.
8. Matsunaga E: Am J Hum Genet 30:406–424, 1978.
9. Vogel F: Hum Genet 52:1–54, 1979.
10. Jensen RD, Miller RW: New Engl J Med 285:307–311, 1971.
11. Abramson DH, Ellsworth RM, Kitchin D, Tung G: Opthalmology 91:1351–1355, 1984.
12. Schimke R, Lowman JT, Cowan GAB: Cancer 34:2077–2079, 1974.
13. Cohen AJ, Li FP, Berg S, Marchetto DJ, Tsai S, Jacobs SC, Brown RS.: New Eng J Med 301:592–595, 1979.
14. Greene MH, Clark WH Jr, Tucker MA, Kraemer KH, Elder DE, Fraser MC: Ann Intern Med 102:458–465, 1985.
15. Knudson AG: Proc Natl Acad Sci USA 68:820–823, 1971.
16. Comings DE: Proc Natl Acad Sci USA 70:3324–3328, 1973.
17. Francke U: Birth Defects 12:131–137, 1976.
18. Yunis JJ, Ramsay N: Am J Dis Child 132:161–163, 1978.
19. Balaban G, Gilbert F, Nichols W, Meadows AT, Shields J: Cancer Genet Cytogenet 6:213–221, 1982.
20. Strong LC, Riccardi VM, Ferrell RE, Sparkes RS: Science 213:1501–1503, 1981.
21. Sparkes RS, Sparkes MC, Wilson MG, Towner JW, Benedict W, Murphree AL, Yunis J: Science 208:1042–1044, 1980.
22. Sparkes RS, Murphree AL, Lingua R, Sparkes MC, Field LL, Funderburk SJ, Benedit WF: Science 219:976–973, 1983.
23. Godbout R, Dryja T, Squire J, Gallie BL, Phillips RA: Nature (London) 304:451–453, 1983.
24. Cavenee WK, Dryja TP, Phillips RA, Benedict WF, Godbout R, Gallie BL, Murphree AL, Strong LC, White RL: Nature (London) 305:779–784, 1983.
25. Benedict WF, Murphree AL, Banerjee A, Spina CA, Sparkes MC, Sparkes RS: Science 219:973–975, 1983.
26. Lee WH, Bookstein R, Wheatly W, Benedict WF, Lee EYHP: Hum Genet 76:33–36, 1987.
27. Dryja TP, Rapaport JM, Joyce JM, Petersen RA: Proc Natl Acad Sci USA 83:7391–7394, 1986.
28. Sager R: Cancer Res 46:1573–1580, 1986.
29. Lee WH, Wheatley W, Benedict WF, Huang CM, Lee EYHP: Proc Natl Acad Sci USA 83:6790–6794, 1986.
30. Lee EYHP, Lee WH: Proc Natl Acad Sci USA 83:6337–6341, 1986.
31. Squire J, Dryja TP, Dunn J, Goddard A, Hofmann T, Musarella M, Willard HF, Becker AJ, Gallie BL, Phillips RA: Proc Natl Acad Sci USA 83:6573–6577, 1986.

32. Lalande M, Dryja TP, Schreck RR, Shipley J, Flint A, Latt, SA: Cancer Genet Cytogenet 13:283–295, 1984.
33. Leppert M, Callahan P, Cavenee W, Holm W, O'Connell P, Thompson K, Lalouel JM, White R: Cytogenet Cell Genet 40:679, 1985.
34. Lee WH, Bookstein R, Hong F, Young LJ, Shew JW, Lee EYHP: Science 235:1394–1399, 1987.
35. Friend SH, Bernards R, Rogelj R, Weinberg R, Rapaport JM, Albert DM, Dryja DP: Nature (London) 323:643–646, 1986.
36. Fung YKT, Murphree AL, T'Ang A, Qian J., Hinrichs SH, Benedict WF: Science 236:1657–1661, 1987.
37. Lee EYHP, Bookstein R, Young LJ, Lin CJ, Rosenfeld MG, Lee WH: Proc Natl Acad Sci USA 85:6017–6021, 1988.
38. Lee WH, Shew JY, Hong FD, Sery TW, Donoso LA, Young LJ, Bookstein R, Lee EYHP: Nature (London) 329:642–645, 1987.
39. Berg JM: Science 232:485–487, 1986.
40. Bookstein R, Lee EYHP, To H, Young LJ, Sery TW, Hayes RC, Friedmann T, Lee WH: Proc Natl Acad Sci USA 85:2210–2214, 1988.
41. Kan W, Dozy AM: Proc Natl Acad Sci USA 75:5631–5635, 1978.
42. Woo SLC, Lidsky AS, Guttler F, Chandra T, Robson KJH: Nature (London) 306:151–155, 1983.
43. DiLella AG, Marvit J, Lasky AS, Guttler G, Woo SLC: Nature (London) 322:7996–803, 1986.
44. DiLella AG, Marvit J, Brayton K, Woo SLC: Nature (London) 327:333–336, 1987.
45. Orkin SH, Kazazian HA: Ann Rev Genet 18:131, 1984.
46. Lehrman MA, Russell DW, Goldstein JL, Brown MS: J Biol Chem 262:3354–3361, 1987.
47. Lehrman MA, Goldstein JL, Russell DW, Brown MS: Cell 48:827–835, 1987.
48. Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM: Cell 50:509–517, 1987.
49. Reid TW, Albert DM, Rabson AS, Russell P, Craft J, Chu EW, Tralka TS, Wilcox JL: J Natl Cancer Inst 53:347–360, 1974.
50. Gasser SM, Laemmli UK: Cell 46:521–530, 1986.
51. Darras BT, Francke U: Nature (London) 329:556–558, 1987.
52. Vanin EF, Henthorn PS, Kioussis D, Grosveld F, Smithies O: Cell 35:701–709, 1983.
53. Weissman BE, Saxon PJ, Pasquale SR, Jones GR, Geiser AG, Stambridge EJ: Science 236:175–180, 1987.
54. Botstein D, Davis, RW: "The Molecular Biology of the Yeast Saccharomyces." Cold Spring Harbor, New York:Cold Spring Harbor Laboratory, 1982.
55. Schatz PJ, Solomon F, Botstein D: Mol Cell Biol 6:3722–3733, 1986.
56. Shortle D, Novick P, Botstein D: Proc Natl Acad Sci USA 81:4889–4983, 1984.
57. De Loanee A, Spudich JA: Science 236:1086–1091, 1987.
58. Thomas KR, Capecchi MR: Nature 324:34–38, 1986.
59. Izant JG, Weintraub H: Cell 36:1007–1015, 1984.
60. Kim SK, Wood BJ: Cell 42:129–138, 1985.
61. Crowley TE, Nellen W, Gomer RH, Firtel RA: Cell 43:633–641, 1985.
62. McGarry TJ, Lindquist S: Proc Natl Acad Sci USA 83:399–403, 1986.
63. Rosenberg UB, Preiss A, Seifert E, Jackle H, Knipple DC: Nature 313:703–706, 1983.
64. Knecht DA, Loomis WF: Science 236:1081–1086, 1984.
65. Yunis JJ: Science 221:227–236, 1983.
66. Hansen MF, Koufos A, Gallie BL, Phillips RA, Fodstad O, Brogger A, Gedde-Dahl T, Cavenee WK: Proc Natl Acad Sci USA 82:6216–6220, 1985.
67. Lundberg C, Skoog L, Cavenee WK, Nordenskjold M: Proc Natl Acad Sci USA 84:2372–2376, 1987.
68. Orkin SH, Goldman DS, Sallan SE: Nature (London) 309:172–174, 1984.
69. Fearon ER, Vogelstein B, Feinberg AP: Nature (London) 309:176–178, 1984.
70. Koufos A, Hansen MF, Lampkin BC, Workman ML, Copeland NG, Jenkins NA, Cavenee WK: Nature (London) 309:170–172, 1984.
71. Koufos A, Hansen MF, Copeland NG, Jenkins NA, Lampkin AC, Cavenee WK: Nature (London) 316:330–334, 1985.
72. Fearon ER, Feinberg AP, Hamilton SH, Vogelstein B: Nature (London) 318:377–380, 1985.
73. Ali RU, Lidereau R, Theillet C, Callahan R: Science 238:185–188, 1987.
74. Broudeur GM, Green AA, Hayes FA, Williams KJ, Williams DL, Tsiatis AA: Cancer Res 41:4678–4686, 1981.

75. Naylor S, Johnson BE, Minna JD, Sakaguchi AY: Nature (London) 329:451–454, 1987.
76. Zbar B, Brauch H, Talmadge C, Lineham M: Nature (London) 327:721–724, 1987.
77. Seizinger BR, Martuza RL, Gusella JF: Nature (London) 322:644–647, 1986.
78. Solomon E, Voss R, Hall V, Bodmer WF, Jass JR, Jeffries AJ, Lucibello FC, Patel I, Rider SH: Nature (London) 328:616–619, 1987.
79. Fearon ER, Hamilton SH, Vogelstein B: Science 238:193–197, 1987.
80. Mendoza A, Shew JY, Lee EYHP, Bookstein R, Lee WH: Hum Pathol 19:487–489, 1988.
81. Wiggs J, Nordenskjold M, Yandell D, Rapaport J, Grondin V: N Engl J Med 318:151–157, 1988.
82. Young LJ, Lee EYHP, To H, Bookstein R, Shew JY, Donoso LA, Sery T, Giblin M, Shields JA, Lee WH: Hum Genet 79:137–141, 1988.
83. Frydman M, Bonne-Tamir B, Farrer LA, Conneally M, Magazanik A, Ashbel S, Golwitch Z: Proc Natl Acad Sci USA 82:1819–1821, 1985.
84. Lee EYHP, To H, Shew JY, Bookstein R, Scully P, Lee WH: Science 241:218–221, 1988.