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Structural Evidence for the Authenticity of the Human Retinoblastoma Gene

YUEN-KAI T. FUNG,* A. LINN MURPHREE, ANNE T'ANG, JIN QIAN, STEVEN H. HINRICHS, WILLIAM F. BENEDICT

The retinoblastoma (Rb) gene is the prototype for a class of recessive human cancer genes in which loss of activity of both normal alleles is thought to be associated with tumorigenesis. Sixteen of 40 retinoblastomas examined with a complementary DNA probe shown to be the Rb gene had identifiable structural changes of the Rb gene including in some cases homozygous internal deletions with corresponding truncated transcripts. An osteosarcoma also had a homozygous internal deletion with a truncated transcript. In addition, possible hot spots for deletion were identified within the Rb genomic locus. Among those tumors with no identifiable structural changes there was either absence of an Rb transcript or abnormal expression of the Rb transcript. Comparison of the structural changes in the tumor cells and fibroblasts of certain patients provided support for Knudson's two-hit hypothesis for the development of retinoblastoma at the molecular level. The ability to detect germline structural deletions in fibroblasts from some patients with bilateral retinoblastoma also indicates that the isolated gene is useful for diagnostic purposes.

RETINOBLASTOMA (Rb) OCCURS IN both hereditary and nonhereditary forms and is the most common childhood cancer involving the eye (*1–4*). Sporadic bilateral cases are always hereditary while approximately 85% of sporadic unilateral cases are nonhereditary. On the basis of statistical analysis of clinical data, Knudson has proposed that the development of retinoblastoma requires two mutational events (*5*). In hereditary cases, where the germline mutation is present in all cells, Knudson postulated that a second random somatic mutation in a target retinoblast is required for tumorigenesis. The initial clue to the nature of the first mutational event came from cytogenetic studies of lymphocytes or fibroblasts from patients with retinoblastoma. A small subgroup of these patients were found to have a deletion including chromosomal region 13q14 (*6*). Initial tumor cytogenetic data also implicated chromosome 13 as the target for the second event. A loss or partial deletion of chromosome 13 including 13q14 was frequently found in retinoblastomas from patients with two apparently normal constitutional No. 13 chromosomes (*7*).

Additional evidence that the second event also involved the Rb susceptibility locus at 13q14 came from restriction fragment length polymorphism (RFLP) studies. By a comparison of the constitutional and tumor genotypes, as defined by RFLPs on chromosome 13, it was shown that the reduction to hemizygosity or homozygosity for all or part of an allele of chromosome 13 is a common event in the development of retinoblastoma (*2, 3*). Similarly, in osteosarcoma, a bone tumor which occurs frequently as an additional primary malignancy in patients with hereditary retinoblastoma (*8*), the development of homozygosity or hemizygosity for a chromosome 13 has been observed (*9*). All the available data suggest that the loss of activity of both normal alleles at the Rb locus is the key event in tumor development (*4*). However, it has not been possible until now to prove this hypothesis with certainty. In this paper, proof is provided by examining the genotypes of fibroblasts and tumor cells from the same individual with a probe of the putative Rb locus.

Our basic strategy of cloning involved chromosomal walking using probes in region 13q14.1, the locus of the Rb gene (*10*). Random probes free of highly repetitive sequences were isolated from the chromosome 13 specific library, LL 13NS01, obtained from the Lawrence Livermore Laboratory. We made use of two hamster-human hybrid cell lines; cell hybrid 3B6 was made with the cell strain 32T, which has a chromosomal deletion from 13q14.1 to 13q14.3 (*11*), whereas 2D12 contains a chromosome 13 with a deletion from 13q14.11 to 13q22.3 (*12*). Clones containing DNA inserts within chromosomal region 13q14.1 to 13q14.3 were identified by the absence of hybridization to DNA from 3B6 and 2D12. Two other probes, H2-42 and H3-8 (*13*), were obtained by similar strategy. In addition, since both the esterase D (EsD) and Rb loci are in 13q14.1 (*10*), we also isolated a full-length EsD complementary DNA (cDNA) clone to use as a probe.

H3-8 was found to be missing from several phage and cosmid libraries, presumably because it is in a region that is easily deleted. We therefore chose to focus on this troublesome region by cloning shorter restriction fragments detected with the H3-8 probe (Fig. 1A). The insert size of each clone obtained was verified by hybridization of the clone to genomic blots.

We were surprised to isolate a probe, PG4-4, which was located very near H3-8

Y.-K. T. Fung, Departments of Pediatrics and Microbiology, USC School of Medicine, Division of Hematology-Oncology, Childrens Hospital of Los Angeles, Los Angeles, CA 90027.
A. L. Murphree, Departments of Ophthalmology and Pediatrics, USC School of Medicine, Childrens Hospital of Los Angeles, Los Angeles, CA 90027.
A. T'Ang and J. Qian, Division of Ophthalmology, Childrens Hospital of Los Angeles, Los Angeles, CA 90027.
S. H. Hinrichs, Department of Pathology, School of Medicine, University of California at Davis, Davis, CA 95616.
W. F. Benedict, Departments of Ophthalmology and Pediatrics, USC School of Medicine, and the Division of Ophthalmology, Childrens Hospital of Los Angeles, Los Angeles, CA 90027.

*To whom correspondence should be addressed.

and which also hybridized to a hamster sequence. This sequence conservation between human and hamster suggested to us that PG4-4 contains the coding sequence of a gene. Hybridization of PG4-4 to a messenger RNA (mRNA) blot of several immortalized human fetal retinal cell lines revealed a 4.7-kb mRNA (Fig. 1B). Using PG4-4 as a probe, we subsequently isolated several cDNA clones the longest of which was 4.73 kb (Fig. 2A). The exon sequence in PG4-4 was found to be located between the Hpa I and Eco RV sites near the 5′ end of the cDNA. Our nucleotide sequencing data show the presence of a GC-rich region between the extreme 5′ end and the Hpa I site. Hybridization of Southern blots to a probe made from this GC-rich region gave a smeared pattern.

If the cDNA clone is the putative Rb gene, we would expect it to detect structural anomalies in the DNA of certain retinoblastomas, including those with visible deletions in the 13q14.1 region. Hind III generated well-separated restriction fragments in normal human DNA that could be detected by subclones of the 4.73-kb cDNA clone; the order of the genomic fragments detected is shown in Fig. 2B. Since the cDNA probe was used, only structural aberrations of the exon-containing restriction fragments would be detectable. Structural aberrations in restriction fragments containing a very small exon might not be detected. This would also be true for point mutations or extremely small deletions in the exon or in splicing junctions. However, even with these limitations we were able to observe structural aberrations including part or all of the Rb gene in many cases. Some of the structural changes which were found in various tumors, including two osteosarcomas, are summarized in Table 1 along with the Rb-specific mRNA expression observed by means of the PG4-4 probe. Additional cases with no detectable changes in the genomic locus but which had either no transcript or transcripts with abnormal sizes are also summarized.

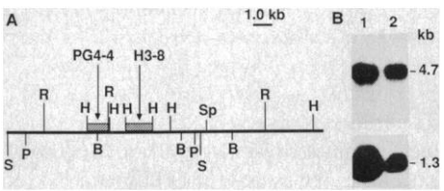

Fig. 1. (**A**) Restriction map of part of the Rb locus at the H3-8 region. An Eco RI 7.7-kb clone was isolated from a genomic library constructed by digesting to completion the genomic DNA of a normal human fibroblast (WSI) with Eco RI and cloned into the Eco RI site of the vector Charon 21A (*18*). Restriction fragments free of repetitive sequence obtained close to the ends of this clone were in turn used as probes to screen a genomic library of Hind III fragments from WSI. Similarly, overlapping clones were screened from genomic libraries constructed from completely digested WSI genomic DNA with Bgl II or Sac I. Restriction enzyme sites are indicated as follows: R, Eco RI; S, Sac I; P, Pst I; B, Bgl II; H, Hind III; Sp, Sph I. (**B**) Northern blot analysis of immortalized human fetal retinal cells. Polyadenylated RNAs (10 μg) were isolated from the human fetal retinal cell lines Ad5/HER B90 and Ad12/HER 8.24, separated by 1% agarose gel electrophoresis (*18*) in 2.2*M* formaldehyde, and transferred to GeneScreen membranes (New England Nuclear). ^{32}P-labeled complementary RNAs were synthesized as described (*20*) with Bam HI linearized PG4-4 and pESD, a subclone of a 1.3-kb human cDNA clone. Random primer probes were synthesized as described (*19*). Hybridization was done at 42°C in 50% formamide, 3× SSC, 1× Denhardt's solution, 0.1*M* Hepes, *p*H 7.4, 0.05% SDS. Washing was done at 2× SSC at room temperature for 20 minutes followed by 0.1× SSC, 0.1% SDS at 65°C twice for 1 hour each. The 4.7- and the 1.3-kb transcripts were detected with the PG4-4 probe and the pESD probe, respectively.

DNA from 40 cases of retinoblastoma obtained at our institution and from fibroblasts of the same patients were examined by Southern blot analysis. To facilitate this analysis, we subcloned the 0.9-kb and 3.8-kb Eco RI fragments (see Fig. 2A) into the vector PGEM-4 (Promega Biotech). Southern blots of genomic DNA isolated from the retinoblastomas digested with different restriction endonucleases were hybridized to probes made from these two subclones, PG H2 (Eco RI; 0.9 kb) and PG 3.8M (Eco RI; 3.8 kb). Polyadenylated RNA from several retinoblastomas was also analyzed with these probes.

One of the mechanisms by which the two Rb alleles are inactivated is the total or partial deletion of both Rb alleles. An example of the total deletion of both alleles is provided by the tumor, LA-RB128B, from a patient with bilateral retinoblastoma. Cytogenetic analysis of fibroblasts from this individual by high resolution banding revealed a visible deletion in one chromosome 13 from 13q14.1 to 13q14.3. The remaining chromosome 13 appeared normal. Consistent with these observations, the intensities of the Rb restriction fragments indicated the presence of only a single copy of an apparently intact Rb locus in the fibroblasts (Fig. 3, A and B, lanes 3). RFLP studies with chromosome 13 probes above and below the 13q14.1 region showed that both chromosome 13s were present in the tumor. However, both alleles of the Rb locus were missing (Fig. 3, A and B, lanes 2). These data imply that in this patient both the first and second mutational event involved deletion of one Rb allele. As expected, no Rb transcripts could be detected in the tumor (Fig. 4, lane 4).

LA-RB165 represents an example of a partial deletion. This tumor was obtained from a patient with unilateral disease. As shown in Fig. 3B, lane 4, the Hind III 9.8-, 6.2-, and 2.1-kb fragments were missing. The intensities of the remaining Rb fragments indicate the existence of only a single copy of the aberrant Rb locus. Thus, in this tumor, one allele of the Rb gene was totally deleted and the other had a deletion at the 3′ end. Two copies of apparently intact Rb loci were present in the fibroblasts (Fig. 3, A and B, lanes 5). This implies that two different mutational events occurred in the somatic cell (the target retinoblast), a result that is consistent with the unilateral (nonhereditary) status of this retinoblastoma patient. No Rb transcript was detected in this tumor (Fig. 4, lane 5).

Two retinoblastomas and one osteosarcoma were found to have homozygous in-

Table 1. Summary of structural changes and expression of the Rb gene in various tumors.

Tumor	Form of disease	Structural change in Rb gene	Expression of Rb gene at mRNA level
LA-RB128B	Deletion	Homozygous total deletion	Absent
LA-RB165	Unilateral	Homozygous 3′ deletion	Absent
LA-RB74	Bilateral	Homozygous internal deletion	Abnormal
LA-RB151	Bilateral	Homozygous internal deletion	ND†
OHS-50	Bilateral*	Homozygous internal deletion	Abnormal
LA-RB125	Unilateral	Internal deletion of one allele	ND
LA-RB99	Unilateral	Internal deletion of one allele	Abnormal
LA-RB112	Unilateral	Total deletion of one allele	ND
LA-RB157	Bilateral	Total deletion of one allele	Absent
OSV	Primary osteosarcoma	Total deletion of one allele	Abnormal
LA-RB69A	Bilateral	None detectable	Absent
LA-RB73	Bilateral	None detectable	Absent
LA-RB110	Bilateral	None detectable	Absent
LA-RB133	Bilateral	None detectable	Absent
LA-RB160	Unilateral	None detectable	Absent
LA-RB94	Unilateral	None detectable	Abnormal
LA-RB70	Unilateral	None detectable	Reduced
Y-79	Bilateral	None detectable	Abnormal

*Osteosarcoma in patient with bilateral retinoblastoma. †Not determined.

ternal deletions. The first example came from a patient with bilateral retinoblastoma, tumor LA-RB74 (Fig. 3B, lane 6), in which the 7.5-kb Hind III restriction fragment was missing. One copy of a new 4.1-kb band could be detected. In this tumor, the Rb transcript was reduced in size to 4.4 kb, consistent with the deletion of all or part of an exon present in the missing 7.5-kb Hind III fragment (Fig. 4, lane 6). Figure 3B, lane 7, shows the restriction pattern of the fibroblasts from the same patient. The presence of one copy each of the normal 7.5-kb and the aberrant 4.1-kb Hind III fragment indicates that this patient has a germline deletion in this region (Fig. 3B, lane 7).

A second example of a homozygous internal deletion was seen in tumor LA-RB151, also from a patient with bilateral retinoblastoma. RFLP studies showed that chromosome 13 probes both above and below the Rb locus were heterozygous in fibroblast cells and became homozygous in tumor cells (*14*). In the tumor both copies of the 9.8-kb Hind III fragment were missing and two copies of a novel 8.0-kb Hind III fragment were present (Fig. 3B, lane 8). Judging from the intensity of the 9.8-kb Hind III fragment in the fibroblasts (Fig. 3B, lane 9) we conclude that only a single copy of the 9.8-kb Hind III fragment was present in the fibroblasts.

A third example of a homozygous internal deletion was found in the osteosarcoma, OHS-50. This tumor was obtained from a patient who had a previous history of retinoblastoma (*15*). The 7.5-kb Hind III fragment was missing (Fig. 3B, lane 10); the small amount of background hybridization to the 7.5-kb fragment presumably came from a few contaminating normal cells. While both OHS-50 and LA-RB74 had a deletion of the 7.5-kb Hind III fragment and truncated transcripts, the Rb gene transcript in OHS-50 was reduced to 3.9 kb (Fig. 4, lane 7), which is smaller than that for LA-RB74 (Fig. 4, lane 6).

In two cases, deletions could be detected in only one allele. Tumor LA-RB125 was from a patient with unilateral sporadic retinoblastoma. Only a single copy of the 7.5-kb Hind III fragment was present, in contrast to two copies of the rest of the Rb gene. Apparently one of the Rb alleles contained an internal deletion in the 7.5-kb region. LA-RB99 was from a unilateral case of retinoblastoma in which an internal deletion involving the Hind III 9.8-kb fragment could be detected in one of the Rb alleles (Fig. 3B, lane 21). A new fragment, also of 7.5 kb, was generated. Two different size transcripts, a 7.0 kb and a normal sized 4.7 kb, could be detected in the tumor (Fig. 4, lane 20). Further analysis is required to determine how this aberrant message is generated. In both of these cases an abnormality could be detected in only one of the chromosome 13s.

Three tumors with total deletion of one of the Rb alleles were also observed (Fig. 3, A and B, lanes 13 to 15). LA-RB112 (lane 13) was from a patient with unilateral sporadic disease, LA-RB157 (lane 14) was from a bilateral case, and OSV (lane 15) is an osteosarcoma from a patient with no known

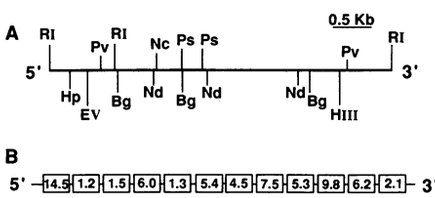

Fig. 2. (**A**) Restriction map of the 4.73-kb cDNA clone of the Rb gene. The 4.73-kb cDNA clone was isolated from a cDNA library constructed as described previously (*21*). Restriction enzyme sites indicated as follows: RI, Eco RI; Hp, Hpa I; EV, Eco RV; Pv, Pvu II; Bg, Bgl II; Nd, Nde I; Nc, Nco I; Ps, Pst I; and HIII, Hind III. (**B**) Linear order of the Hind III restriction fragments detectable with the cDNA probes. Each of the boxes represents a discrete Hind III fragment the size (kilobases) of which is indicated by the number in the box. The order of these fragments was deduced partly from Southern blot hybridization and from restriction mapping of phage clones isolated from a normal human genomic library.

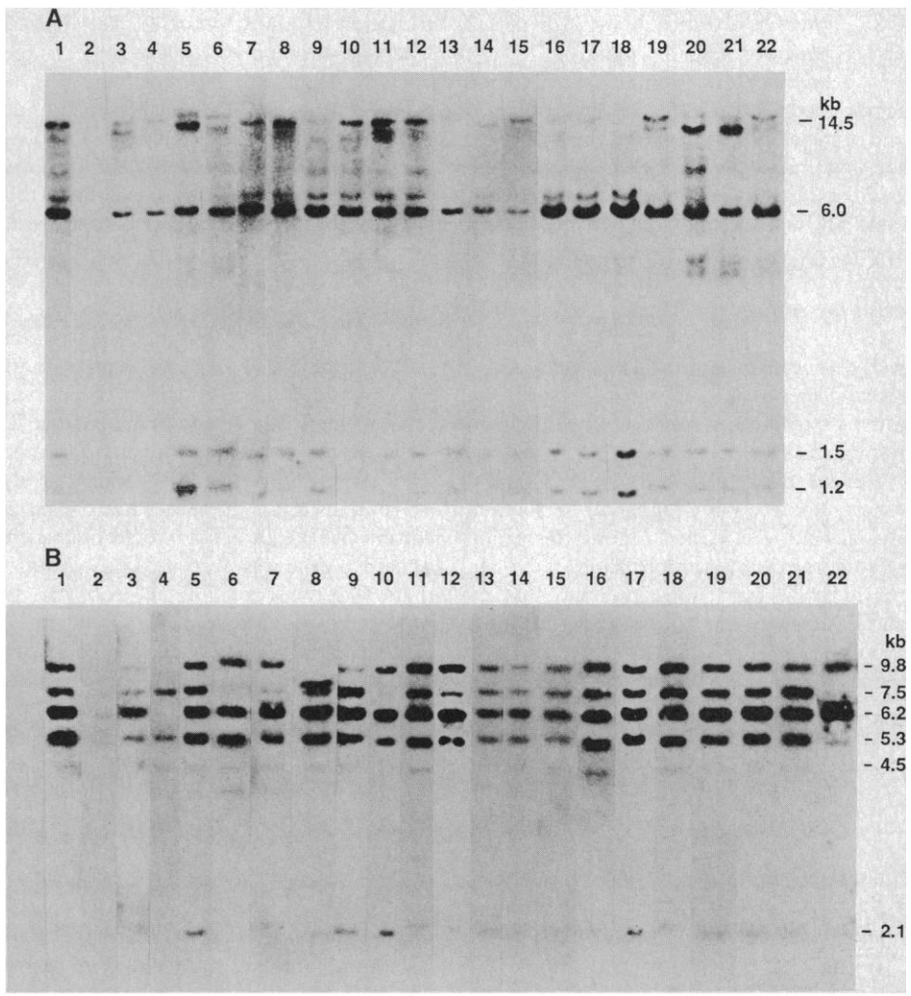

Fig. 3. Southern blot analysis of Hind III genomic DNA from retinoblastoma and their constitutional cells and normal cells. (**A**) Hybridization to PGH2 (a subclone of the Eco RI 0.9-kb fragment at the 5′ end of the cDNA clone). (**B**) Hybridization to PG 3.8M (a subclone of the Eco RI 3.8-kb fragment of the cDNA clone). Samples in each lane for both (A) and (B) are identical. Lanes 1, normal control human fibroblast; lanes 2, LA-RB128B (tumor); lanes 3, LA-RB128B-F (fibroblast); lanes 4, LA-RB165 (tumor); lanes 5, LA-RB165-F (fibroblast); lanes 6, LA-RB74 (tumor); lanes 7, LA-RB74-F (fibroblast); lanes 8, LA-RB151 (tumor); lanes 9, LA-RB151-F (fibroblast); lanes 10, OHS-50 (osteosarcoma); lanes 11, LA-RB73 (tumor); lanes 12 LA-RB125 (tumor); lanes 13, LA-RB112 (tumor); lanes 14, LA-RB157 (tumor); lanes 15, OSV (osteosarcoma); lanes 16, LA-RB94 (tumor); lanes 17, LA-RB70 (tumor); lanes 18, Y-79 (tumor); lanes 19, LA-RB69A (tumor); lanes 20, LA-RB69A-F (fibroblast); lanes 21, LA-RB99 (tumor); lanes 22, RB120F (fibroblast that has a balanced translocation 46, XX, t[10, 13] [q22; q14]).

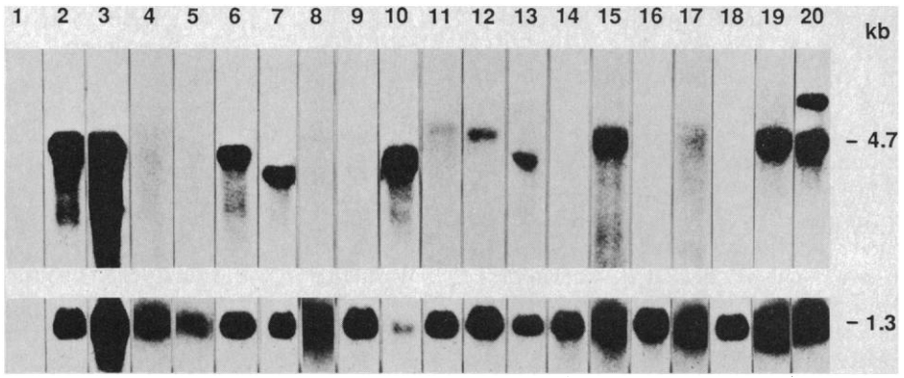

Fig. 4. Northern blot analysis of polyadenylated RNA (10 μg) from normal human fibroblast, normal and immortalized human retinal cells, retinoblastoma and their fibroblasts. The isolation and hybridization conditions were as described in Fig. 1. Normal human retinal cells were isolated from eight 14- to 16-week abortuses. Lane 1, poly(A)$^+$RNA; lanes 2, normal human fetal retinal cells; lane 3, Ad5/HER A (adenovirus 5 immortalized human retinal cells); lane 4, LA-RB128B; lane 5, LA-RB165; lane 6, LA-RB74; lane 7, OHS-50; lane 8, LA-RB73; lane 9, LA-RB157; lane 10, OSV; lane 11, LA-RB94; lane 12, LA-RB70; lane 13, Y-79; lane 14, LA-RB69A; lane 15, LA-RB69A-F (fibroblast); lane 16, LA-RB133; lane 17, LA-RB110; lane 18, LA-RB160; lane 19, WS 1 (fibroblast); lane 20, LA-RB99.

history of retinoblastoma. In all these cases, only a single, apparently normal, Rb locus remained. Little or no Rb transcript was detected in LA-RB157 (Fig. 4, lane 9). In OSV, however, a truncated message of 4.2 kb was observed (Fig. 4, lane 10).

The presence of a stretch of a GC-rich region (greater than 82%) at the extreme 5′ end of the cDNA complicated the analysis of the genomic locus. If we assume that the 14.5-kb Hind III fragment (Fig. 2B) contains the first exon of the Rb gene, then seven cases of retinoblastoma not shown here have 5′ deletions. If, however, there exists yet another small exon in front of the 14.5-kb Hind III fragment then these cases would represent additional examples of internal deletions. Cloning and sequencing of this part of the genomic locus will help to resolve this issue.

Approximately 60% of the 40 retinoblastomas showed no apparent structural changes in the Rb gene. Among this group of tumors, various abnormalities were observed in Rb transcript expression. For example, none of the following five tumors expressed an Rb transcript: the bilateral retinoblastomas LA-RB73 (Fig. 4, lane 8), LA-RB69A (Fig. 4, lane 14), and LA-RB133 (Fig. 4, lane 16), all of which are homozygous for a chromosome 13 (*14*); the bilateral tumor LA-RB110 (Fig. 4, lane 17), which is heterozygous for chromosome 13 polymorphic probes both above and below the Rb locus; and the unilateral tumor LA-RB160 (Fig. 4, lane 18), which has become homozygous for a 13q14 probe, 7D2 (*14*).

In other cases, abnormal transcripts were also detected. For example, the unilateral case, LA-RB94, which is homozygous for chromosome 13 (*14*), shows no gross structural changes (Fig. 3, A and B, lanes 16) but has a small amount of an apparently larger Rb transcript (Fig. 4, lane 11). This presumably is due to the loss of a splice junction such that a small intron is included in the final transcript. LA-RB70, which also has no apparent change in the Rb DNA locus, (Fig. 3, A and B, lanes 17) has a reduced amount of a normal size Rb transcript (Fig. 4, lane 12). Finally, Y79, the most commonly used retinoblastoma cell line to date, has consistently shown a reduced amount of a truncated Rb transcript of 4.2 kb (Fig. 4B, lane 13), although no gross DNA changes could be observed with our probes (Fig. 3, A and B, lanes 18). Presumably, a yet undetected deletion has occurred in the Rb locus.

Recently, Friend *et al.* (*16*) and Lee *et al.* (*17*) reported the isolation of a partial cDNA clone of the Rb gene by means of the same intron probe, H3-8, that we used. Judging from the restriction maps, data from the Southern blot analyses, and nucleotide sequencing, the two reported cDNAs are essentially shorter versions of the one reported here. While their observations of deletions involving either the 3′ end of the putative Rb locus or the entire gene in retinoblastoma were supportive of the contention that the 4.7-kb transcript is the authentic Rb gene, an equally viable explanation would be that the actual Rb gene was 3′ to the locus encoding the 4.7-kb transcript. Formal proof that the 4.7-kb transcript is indeed encoded by the Rb locus requires the demonstration of internal homozygous deletions or 5′ deletions in other retinoblastomas. Our data on homozygous internal deletions at the DNA level and the corresponding aberrant transcript provided this proof and confirmed that the 4.7-kb transcript was the Rb susceptibility gene (Table 1). In support of this conclusion, we observed that while abundant amounts of normal Rb transcript can be detected in normal human fetal retinal cells none of the 15 retinoblastomas shown in Fig. 4 had normal Rb transcription (Fig. 4 and Table 1).

A homozygous internal deletion and truncated Rb transcript were also observed in an osteosarcoma from a patient with a prior history of retinoblastoma. These data are consistent with the postulate that the Rb gene is responsible for the development of both retinoblastoma and osteosarcoma. The observation that the majority of the tumors do not express the Rb gene at the RNA level provide strong support for the recessive nature of the Rb gene and the contention that it is the loss of function of both Rb alleles which is responsible for tumor development (*4*).

In all the internal deletion cases examined, deletions always involved either the 7.5- or 9.8-kb Hind III fragments. One copy of the Hind III 7.5-kb fragment was also found to be altered (Fig. 3B, lane 22) in the RB120F fibroblasts. This fibroblast cell line was from a patient with bilateral retinoblastoma in which a balanced translocation involving chromosomal region 13q14 and 10q22 had occurred. A novel band of 6.5 kb was detected instead. Whether these regions of the Rb gene locus contain peculiar structures that would predispose them to deletion or translocation remains to be examined.

Our data (LA-RB128B, LA-RB74, and LA-RB151) also provided a verification of Knudson's two-hit hypothesis (*5*). In bilateral cases a mutation at the Rb locus representing the first hit was readily detected in the fibroblasts (Fig. 3B, lanes 3, 7, and 9) and the second hit was found in the tumor (Fig. 3B, lanes 2, 6, and 8). In the unilateral case, LA-RB165, the two mutational events (3′ deletion and total deletion) were seen only in the tumor (Fig. 3B, lane 4) and not in the fibroblasts (Fig. 3B, lane 5).

The fact that structural changes within the Rb locus could be readily detected with our cDNA clone in approximately 40% of the retinoblastomas we examined as well as in fibroblasts from some bilateral patients is quite encouraging for its potential use in prenatal diagnosis. When the complete genomic locus is analyzed and better probes and methodologies are available, considerably more abnormalities should be detected. Nonetheless, this cDNA probe should be immediately useful in prenatal diagnosis in families having a parent with a detectable structural abnormality of the Rb gene such as those individuals from whom tumors LA-RB74, LA-RB151, and LA-RB128B or the fibroblast line RB120F were derived. If similar changes were found in fetal cells, the

fetus would have inherited the Rb susceptibility gene. Such a fetus almost certainly would develop retinoblastoma and be at much higher risk to have other malignancies, particularly osteosarcoma, in later years if the pregnancy reaches term. The cDNA probe could also allow one to identify some of the estimated 15% of individuals who have unilateral sporadic Rb but who unfortunately have the hereditary form of the disease.

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Genetic Analysis of the Human Malaria Parasite *Plasmodium falciparum*

DAVID WALLIKER,* ISABELLA A. QUAKYI, THOMAS E. WELLEMS, THOMAS F. MCCUTCHAN, ANA SZARFMAN, WILLIAM T. LONDON, LYNN M. CORCORAN, THOMAS R. BURKOT, RICHARD CARTER*

Malaria parasites are haploid for most of their life cycle, with zygote formation and meiosis occurring during the mosquito phase of development. The parasites can be analyzed genetically by transmitting mixtures of cloned parasites through mosquitoes to permit cross-fertilization of gametes to occur. A cross was made between two clones of *Plasmodium falciparum* differing in enzymes, drug sensitivity, antigens, and chromosome patterns. Parasites showing recombination between the parent clone markers were detected at a high frequency. Novel forms of certain chromosomes, detected by pulsed-field gradient gel electrophoresis, were produced readily, showing that extensive rearrangements occur in the parasite genome after cross-fertilization. Since patients are frequently infected with mixtures of genetically distinct parasites, mosquito transmission is likely to provide the principal mechanisms for generating parasites with novel genotypes.

NEW METHODS ARE NEEDED FOR treating and controlling *Plasmodium falciparum*, the most pathogenic species of malaria parasite infecting humans. Control by chemotherapy has been complicated by the emergence of drug-resistant forms (*1*). Several antigens have been identified as candidates for vaccines but may exhibit considerable diversity in the parasite population (*2*). There is thus a risk that if vaccines based on such antigens are put into widespread use, new or alternative forms of the parasite will be selected and replace previously existing forms.

Few studies have been made on the basic genetics of malaria parasites by conventional strain hybridization techniques. This is mainly because the organisms undergo a complex life cycle in which asexual multiplication and gametocyte development occur in the vertebrate host, while fertilization between gametes takes place in the mosquito vector. Crossing experiments have been possible to date only with species that infect rodents (*P. yoelii* and *P. chabaudi*) (*3*), because the complete life cycle of these parasites can be maintained in the laboratory more easily than most other malaria species. Crosses between rodent parasites have been made by feeding mosquitoes on mixtures of two cloned lines to allow cross-fertilization to occur and then infecting rodents with the resulting sporozoites. The blood forms developing in these animals are then examined for the presence of forms exhibiting recombination between parent clone markers. These studies show that (i) the parasites appear to undergo a normal Mendelian pattern of inheritance of genetically determined characters; (ii) the blood forms are haploid, meiosis probably occurring during early division of the zygote (*4*); (iii) resistance to drugs such as pyrimethamine and chloroquine is due to gene mutations (*5*); and (iv) recombination between genes determining characters such as enzymes, antigens, and drug sensitivity occurs readily after cross-fertilization between clones (*3*).

The asexual erythrocytic forms (*6*) and gametocytes (*7*) of *P. falciparum* can be maintained in culture in vitro. Cultured gametocytes are infective to mosquitoes (*7*), and the resulting sporozoites infective to primates (*8*). Blood forms of *P. falciparum* can be cloned by limiting dilution (*9*) or micromanipulation (*10*), so that genetically pure lines can be obtained. Here we report successful crossing of two cloned lines of *P.*

D. Walliker, I. A. Quakyi, T. E. Wellems, T. F. McCutchan, R. Carter, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.
A. Szarfman, Naval Medical Research Institute and Uniformed Services University of Health Sciences, Bethesda, MD 20814.
W. T. London, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892.
L. M. Corcoran, Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia.
T. R. Burkot, Papua New Guinea Institute of Medical Research, Post Office Box 378, Madang, Papua New Guinea.

*Present address: Department of Genetics, University of Edinburgh, West Mains Road, Edinburgh EH9 3JN, Scotland.