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Molecular detection of deletions involving band q14 of chromosome 13 in retinoblastomas

(oncogene)

THADDEUS P. DRYJA*†, JOYCE M. RAPAPORT*, JENNIFER M. JOYCE*, AND ROBERT A. PETERSEN‡

*Taylor R. Smith Laboratory, Howe Laboratory of Ophthalmology, Massachusetts Eye and Ear Infirmary, 243 Charles Street, Boston, MA 02114; and ‡Department of Ophthalmology, Children's Hospital Medical Center, 300 Longwood Avenue, Boston, MA 02115

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ABSTRACT **DNA fragments from a locus spanning 29 kilobases within chromosome band 13q14 detected deletions in 3 retinoblastomas out of 37 such tumors examined. Somatically occurring, homozygous deletions spanning at least 25 kilobases were detected in retinoblastomas from two unrelated patients. These deletions are bounded by the esterase D locus proximally. In a third patient, both tumor cells and leukocytes have a deletion of one chromosome 13 homolog, with one end of the deletion localized to a 1.55-kilobase fragment within the cloned region. It is likely that the cloned locus is within a few hundred kilobases of the retinoblastoma gene (i.e., the locus governing predisposition to such tumors) and that the deletions detected also involve the retinoblastoma gene. Further, it may be possible to base a successful approach to the isolation of the retinoblastoma gene on this assumed physical proximity of the two loci.**

It has been about 80 years since the appearance of published reports of families in which a parent who was cured of retinoblastoma had children afflicted with the same type of tumor (ref. 1, p. 336; ref. 2). There is evidence from more recent studies that the heritability of predisposition to retinoblastoma is governed by a locus within human chromosome band 13q14 (3–8). Furthermore, the locus is representative of a class of such loci in the human genome, called recessive oncogenes (9, 10). The loci are related, since it appears that recessive, mutant alleles at any of the loci tend to be oncogenic and, correspondingly, that the dominant alleles normally present at the loci have a role in preventing tumor formation (11–16). The evidence supporting this genetic nature of alleles at the retinoblastoma locus has been indirect; i.e., the locus has not been isolated for direct molecular study.

A proportion of the recessive mutations at the locus that predispose to tumor formation are deletions (3, 4). Since most, if not all, retinoblastomas arise from cells that have lost both normal, dominant, homologous alleles at the retinoblastoma locus, we hypothesize that a proportion of tumors are homozygous for deletions affecting the locus. To test this reasoning, we used cloned probes known to detect loci within band 14 of the long arm of chromosome 13 (13q14) to search for deletions in retinoblastomas derived from enucleated eyes. To our satisfaction, we found two retinoblastomas with homozygous deletions within 13q14 including at least 25 kilobases (kb) surrounding the locus defined by hybridization probe pH3-8 (H3-8 locus; see ref. 17). A third tumor was documented to have a deletion of one chromosome 13 homolog, with one end of the deletion occurring within a 1.55-kb fragment approximately 13 kb from the H3-8 fragment. This article reports the data supporting the existence of homozygous deletions within 13q14 occurring in retinoblastomas, as well as evidence pertaining to the genetic origin of these deletions.

MATERIALS AND METHODS

Origin of Probes. There are three loci established to be within 13q14: the esterase D locus and the loci detected by probes pH3-8 and pH2-42 (4, 17). Probes pH3-8 and pH2-42 were derived from a phage library enriched for DNA sequences from chromosome 13 by using a fluorescence-activated chromosome sorter (17). Esterase D is an enzyme, of unknown biologic function, that demonstrates allelic isozymes (18). One other locus, named 7D2, is closely linked to the esterase D locus and is probably within or near 13q14 (19).

Other probes that detect loci assigned to chromosome 13 and were used in this study are p7F12, p9D11, p1E8, p9A7, pHU10, pHU26, and pHUB8 (20, 21). The sublocalization of the loci detected by these probes has been determined by deletion mapping (20, 21) and by *in situ* hybridization (22). Probe p4-A detects an arbitrary autosomal locus not on chromosome 13.

Construction of Phage Library. For the purpose of "chromosome walking," a total human phage library was constructed. Normal human lymphoblast DNA was digested partially with *Mbo* I, with due regard to the calculations of Seed *et al.* (23, 24). The digested fragments were separated by electrophoresis in a low-melting-point agarose gel, and the fragments between 16 and 22 kb in length were recovered with an Elutip-D column (Schleicher & Schuell). Aliquots of the DNA fragments were ligated to EMBL3 phage arms and packaged *in vitro* (25).

Isolation of Phage with Overlapping Inserts. The human phage library was plated directly (no library amplification) on 22 × 22-cm plates at a density of 100,000 colonies per plate. Phage from 10 plates were blotted onto duplicate nitrocellulose filters according to published methods (26). The filters were baked at 80°C for 2–4 hr in a vacuum oven, followed by prewashing in 50 mM Tris Cl, pH 8.0/1 M NaCl/1 mM EDTA/0.1% $NaDodSO_4$ at 65°C for 2–4 hr. They were prehybridized overnight at 68°C in 4× SSC/50 mM sodium phosphate, pH 7.0/0.1% $NaDodSO_4$/1 mM EDTA/0.04% polyvinylpyrrolidone/0.04% Ficoll/0.04% bovine serum albumin/5% dextran sulfate/tRNA (100 μg/ml)/denatured salmon sperm DNA (25 μg/ml). (1× SSC is 0.15 M NaCl/15 mM sodium citrate, pH 7.) The DNA on the filters was subsequently hybridized to the appropriate radiolabeled DNA probes in a fresh batch of the same solution used for prehybridization. After incubation at 68°C overnight, the filters were washed 6–10 times in 0.2× SSC/0.1% $NaDodSO_4$ at room temperature for 5–10 min per wash. The filters were

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Abbreviation: kb, kilobase(s).

†To whom reprint requests should be addressed.

exposed to x-ray film with an intensifying screen for 20–48 hr at −70°C.

Phage plaques hybridizing to selected probes were plaque-purified and amplified according to standard methods. A restriction map of each phage insert was determined primarily through the use of radiolabeled *cos* probes (27). A number of single-copy fragments from the phage inserts were subcloned in plasmid pBR322 or bacteriophage M13.

Analysis of Retinoblastoma and Leukocyte Samples. All retinoblastoma samples included in this study were obtained from tumor fragments retrieved from enucleated eyes. In most cases, including tumors 1, 9, and 27, DNA was isolated directly from these primary tumor fragments. In a few cases, the DNA was derived from established cell lines. In cases 1, 9, and 27, leukocyte DNA from the patients who developed these tumors was isolated. Fragments of two osteosarcomas were obtained also from patients who had no previous history of retinoblastoma. DNA isolation, restriction endonuclease digestion of DNA samples, agarose gel electrophoresis, Southern blotting, and hybridization were all performed according to published methods (28). Analysis of esterase D from tumor fragments was carried out by starch gel electrophoresis (18).

RESULTS

Detection of Deletions of the H3-8 Locus. DNA samples from 37 retinoblastomas from unrelated patients were tested for the presence of sequences hybridizing to the probes pH3-8, pH2-42, and p7D2. Two tumors, nos. 9 and 27, were found to lack the H3-8 locus. These two tumors had esterase D activity detected by starch gel electrophoresis. None of the tumors from the entire set had a homozygous deletion affecting the H2-42 locus or the 7D2 locus. Fig. 1 *Upper* illustrates the absence of the H3-8 locus in tumor 9, as well as the presence of at least one copy of the 7D2 and H2-42 loci in tumors 1–22. Fig. 1 *Lower* shows the absence of the H3-8 locus in tumor 27. A very faint signal, possibly indicating a small amount of DNA hybridizing to H3-8, is barely visible in the DNA derived from tumors 9 and 27 and probably represents DNA from constitutional cells contaminating the fresh tumor samples from which the DNA was isolated.

Chromosome "Walk" from the H3-8 Locus. A total human genomic library constructed in the vector EMBL3 was screened with probe H3-8 to isolate surrounding DNA sequences. One phage, $\phi 7$, was isolated and mapped. A single-copy fragment from one end of the human insert from $\phi 7$ (probe p7RV1.55L) was used to rescreen the library. Three additional overlapping phage inserts were isolated, and a restriction map was determined (Fig. 2). Single-copy fragments from these inserts were subcloned in pBR322 or M13. Tumors 9 and 27, which had homozygous deletions of the H3-8 locus, had deletions of every tested single-copy fragment from these four overlapping phage inserts, including subclones p23RI1.02 and p7H30.7R, which are separated by 25 kb. Hence, the homozygous deletions in these tumors span at least 25 kb of the 29 kb isolated.

Detection of a Heterozygous Deletion near the H3-8 Locus. The single-copy subclones from the phage inserts were used to rescreen the initial set of 37 tumors for additional 13q14 deletions. DNA from tumor 1 digested with either *Hin*dIII, *Eco*RV, *Bgl* II, *Xba* I, *Sac* I, *Mbo* I, *Kpn* I, or *Xmn* I was found to have an extra hybridizing fragment compared to lymphoblast DNA derived from normal individuals, when probed with p7RV1.55L (Fig. 3). Tumor 1 DNA digested with *Kpn* I also has an extra hybridizing fragment when probed with pH3-8 or p7H30.7R, but not with p23RI1.02. Furthermore, dosage blots of tumor 1 DNA compared to normal lymphoblast DNA are consistent with tumor 1 having two copies of the H3-8 locus, but one copy of the p23RI1.02 locus

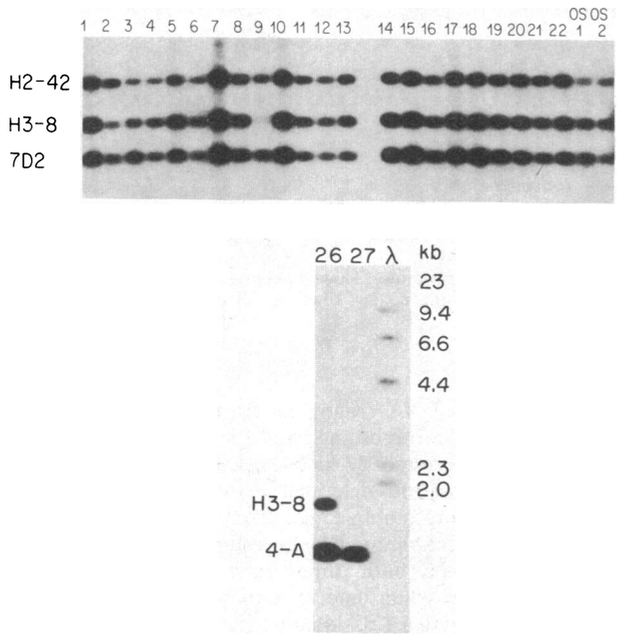

FIG. 1. (*Upper*) DNA from retinoblastomas 1–22, as well as DNA from two osteosarcomas (OS1 and OS2), digested with *Hin*dIII and probed simultaneously with pH2-42, pH3-8, and p7D2. No DNA is present in the lane between lanes 13 and 14. Note that pH3-8-hybridizing fragments are not present in lane 9, which contains DNA from retinoblastoma 9. None of the other tumors in this panel has a homozygous deletion of any of the three probe loci. (*Lower*) DNA from retinoblastomas 26 and 27, digested with *Hin*dIII and probed simultaneously with pH3-8 and p4-A. Probe 4-A recognizes a random autosomal locus not on chromosome 13 and serves as a control probe in this blot. Note that there is an absence of fragments hybridizing to the H3-8 probe in DNA derived from tumor 27. Lane at right shows marker fragments (sizes in kb) produced by digestion of λ phage DNA with *Hin*dIII.

(data not shown). One can infer that there is a deletion involving one chromosome 13 homolog in tumor 1. One end of the deletion is within the 1.55-kb fragment p7RV1.55L, and the deletion extends to the left-hand side of the restriction map in Fig. 2.

Origin of 13q14 Deletions in Tumors 1, 9, and 27. Leukocyte DNA from the patients who developed tumors 1, 9, and 27 was tested at polymorphic loci assigned to chromosome 13. Leukocytes from patient 1 [referred to as P83-6 in an earlier report (12)] are heterozygous at the loci detected by probes p7F12 [assigned to 13q12–13 (22)], pHU10 (13q13), and p9D11 (13q22). Tumor cells from this patient are also heterozygous at these loci. Hence, both chromosome 13 homologs are present in this tumor. Analysis with the probe

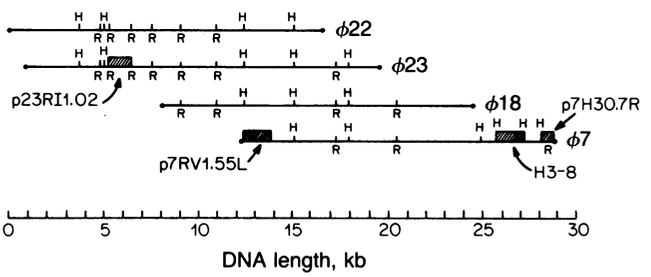

FIG. 2. Restriction map of the region neighboring the H3-8 locus. The map shows the locations of the *Hin*dIII (H) sites and the *Eco*RI (R) sites in the overlapping human inserts of phage numbered $\phi 22$, $\phi 23$, $\phi 18$, and $\phi 7$. Also shown are the locations of the single-copy probes p23RI1.02, p7RV1.55L, pH3-8, and p7H30.7R.

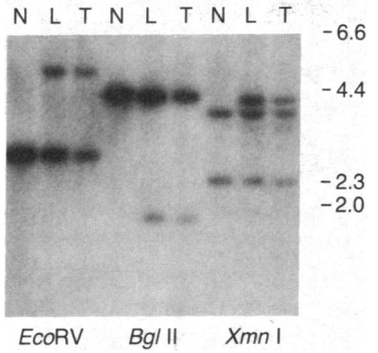

FIG. 3. DNA from a lymphoblast line derived (by Epstein–Barr virus transformation) from a healthy human [lanes N ("normal")], from leukocytes from retinoblastoma patient 1 (lanes L), and from tumor cells from patient 1 (lanes T) was digested with restriction endonucleases *Eco*RV, *Bgl* II, or *Xmn* I and probed with p7RV1.55L. When compared with the normal human DNA, an additional hybridizing fragment is observed in both the leukocyte DNA and the tumor cell DNA from patient 1, regardless of which endonuclease was used to digest the samples. Endonucleases *Hin*dIII, *Mbo* I, *Xba* I, *Sac* I, and *Kpn* I gave a similar result with probe p7RV1.55L. Numbers at right indicate positions of molecular size (kb) markers derived from a *Hin*dIII digest of λ phage DNA.

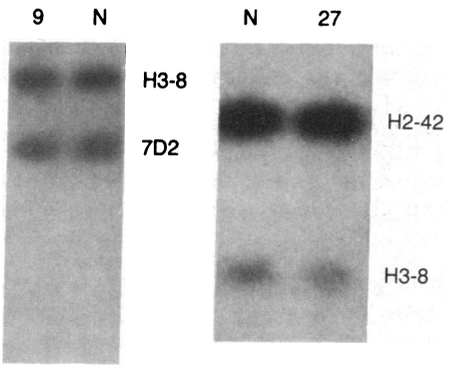

FIG. 4. Dosage blots indicating the presence of two copies of the H3-8 locus in leukocytes from patients 9 and 27. DNA derived from the appropriate patients' leukocytes and from leukocytes from a healthy human (lanes N) were digested with *Hin*dIII. Equal amounts of the digested DNA samples were loaded into adjacent lanes of an agarose gel. After electrophoresis and Southern blotting, the DNA was probed with pH3-8 as well as p7D2 (*Left*) or pH2-42 (*Right*). Equal hybridizing intensity in the adjacent lanes implies equal copy number of the H3-8 locus in the samples under comparison.

p7RV1.55L, whose locus bridges one end of the deletion in tumor 1, reveals extra restriction fragments in leukocytes from patient 1, with sizes identical to those found in this patient's tumor (Fig. 3). Hence, a deletion affecting the q14 band from one chromosome 13 homolog is present in leukocyte DNA, tumor DNA, and probably all constitutional cells from this patient. The extra fragments detected by p7RV1.55L are not present in leukocyte DNA derived from this patient's parents, who had no retinoblastoma (data not shown). Therefore, patient 1 received this mutation germinally. The fact that patient 1 had bilateral retinoblastoma is consistent with the interpretation that this deletion represents a germinal mutation affecting the retinoblastoma locus.

With regard to patient 9 (referred to as patient P82-15 in ref. 12), leukocytes from this patient are heterozygous at the loci detected by probes p7F12 (13q12–13), pHU10 (13q13), and p9D11 (13q22) (12). Tumor cells from this patient also are heterozygous at these three loci, which implies that the tumor cells retain each of the patient's two chromosome 13 homologs. Furthermore, dosage blots reveal that leukocytes do not have a deletion of either of the two homologous H3-8 loci (Fig. 4). This implies that the mechanisms giving rise to the homozygous deletion in this tumor were somatic, which is consistent with the fact that this patient had unifocal retinoblastoma and no family history of the tumor. The development of homozygosity for the detected deleted region may have been the result of two independent, overlapping 13q14 deletions, or of a somatic 13q14 deletion occurring on one chromosome 13 homolog followed by a gene-conversion event deleting the homologous locus.

As with patient 9, leukocytes from patient 27 had two copies of the H3-8 locus, as determined from dosage blots (Fig. 4). Hence, the development of the deletion of the H3-8 locus was somatic. As would be expected for a somatic mutation affecting the retinoblastoma locus, the patient had unifocal retinoblastoma and no family history of the tumor. Unlike patient 9, however, the mechanism for the development of homozygosity for the deletion in the tumor was different. The following loci were heterozygous in leukocytes but homozygous in tumor cells from patient 27: 7F12 (13q12–13), HU10 (13q13), 1E8 (13q22–31), and 9A7 (13q33–34). These data are consistent with a scenario where a deletion including the H3-8 locus occurred somatically on one chromosome 13 homolog and was followed by a loss of all or the majority of the remaining chromosome 13 homolog, presumably by one of a number of possible mitotic mechanisms previously described (11).

Limits to the Size of the Deletions. Although karyotypes were not determined for tumors 9 and 27, some limits to the size of the homozygous deletions present in those tumors can be inferred from the analysis of loci assigned to 13q. Since the esterase D locus is thought to be proximal to the retinoblastoma locus within 13q14 (29), and since tumors 9 and 27 had esterase D activity detectable by starch gel electrophoresis, one may deduce that the deletions present in these tumors are bounded proximally by the esterase D locus. Distally, the loci homologous to probes pHU26 and p9D11, both assigned to 13q22, are present in these two tumors, and therefore the deletions are bounded by these loci.

With regard to the deletion present in patient 1, it can be deduced that this deletion is completely contained within the interval 13q13–22. This is based on the (previously mentioned) facts that the patient's leukocytes and tumor cells are heterozygous at the pHU10 locus within 13q13 and at the p9D11 locus within 13q22.

DISCUSSION

The single-copy probes generated from the region surrounding the H3-8 locus provide a means to detect, by molecular hybridization, deletions affecting 13q14. Genetic changes involving 13q14 have been implicated in the etiology of retinoblastoma and osteosarcoma (35). One previous report (30) describes a homozygous deletion of the esterase D locus in a retinoblastoma, but that determination was based on the absence of esterase D activity in tumor cells. The probes described here avoid the potential shortcomings in diagnosing chromosomal aberrations based on abnormal levels of enzyme activity. Our data allow one to conclude with confidence that deletions involving 13q14 are present in at least some retinoblastomas and that the deletions are occasionally homozygous.

One may consider the hypothesis that the deletions found in the tumors 1, 9, and 27 represent incidental polymorphisms that are present in a proportion of the normal population and are of no consequence with regard to the etiology of retinoblastoma. The argument against this hypothesis is based on the following: (*i*) the origin of the deletions was somatic or germinal and (*ii*) the deletion present in patient 1 is easily detectable with probe p7RV1.55L, yet it has not been

found in a sample of 20 unrelated individuals. It is more likely that the deletions detected in these three retinoblastomas do involve the retinoblastoma locus and were instrumental in the genesis of these tumors.

Previous studies have documented that ≈50% of retinoblastomas arise from cells that have become homozygous at loci distributed along 13q (11, 12). These findings have been used to support a theory that recessive mutations at the hereditary retinoblastoma locus assigned to 13q14 predispose to the tumor. Retinoblastomas arise from sensitive retinal cells that have lost both normal, dominant alleles at the retinoblastoma locus. The first step in oncogenesis is the acquisition of a recessive mutation of one of the two homologous alleles at the retinoblastoma locus, either by inheritance, by germinal mutation, or by somatic mutation. In ≈50% of tumors, homozygosity for such a recessive mutation is accomplished through the mitotic loss of a portion (including the 13q14 band) of the chromosome 13 homolog with a remaining normal allele at the retinoblastoma locus. The resulting homozygosity for the recessive, mutant allele at the locus allows the genesis of the tumor. It has been assumed that the remaining 50% of tumors, which do not show a generalized homozygosity of 13q, nevertheless lose the remaining normal allele at the retinoblastoma locus by some localized mechanism. Tumor 9 from this series supports this reasoning. In this tumor there is a homozygous deletion within 13q14 affecting the retinoblastoma locus, despite the fact that loci located proximally and distally remain heterozygous. Moreover, that the mutations in this case, as well as tumors 1 and 27, are deletions is consistent with the recessive nature theorized for tumorigenic mutations at the retinoblastoma locus.

It is likely that the cloned region described in this paper is in close proximity to the retinoblastoma locus. The incidence of deletions in retinoblastomas affecting the locus (3 out of 37 tumors from unrelated individuals) is in reasonable agreement with deletions as a cause of recessive mutation at other human loci, such as the Duchenne muscular dystrophy locus (31, 32), the hypoxanthine phosphoribosyl transferase locus (33), and the α- and β-globin loci (34). We predict that any restriction fragment length polymorphisms revealed by the single-copy probes from this region would be closely linked to the retinoblastoma locus and would be helpful in the genetic counseling of members of kindreds with hereditary retinoblastoma.

The homozygous deletions found in tumors 9 and 27 are bounded proximally by the esterase D locus and, hence, do not extend outside of the proximal limit of 13q14. Their distal termination is still unknown. It may be that the cloned locus is within "chromosome walking" distance of the retinoblastoma locus. We suggest that a feasible approach to the isolation of the retinoblastoma gene can be based on this probable close proximity.

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