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Original investigations

A detailed analysis of chromosomal changes in heritable and non-heritable retinoblastoma

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Summary. Full cytogenetic analysis of 27 different retinoblastoma tumors is presented. Gross aneuploidy of chromosome arms 6p and 1q were very common, being observed in 15/27 and 21/27 tumors, respectively. However, we found that chromosome 13 was rarely missing: only 3/27 had a detectable monosomy affecting 13q14. Monosomy of chromosome 13 by small deletion or rearrangement was also not observed in any of 12 retinoblastoma tumor lines analyzed detail at the 300–400 chromosome band level. A novel observation in retinoblastoma was the discovery of non-random translocations at three specific breakpoints, 14q32 (4/12), 17p12 (5/12), and 10q25 (3/12). Genomic rearrangements similar to those described involving C-myc in Burkitt lymphoma 14q+ cells could not be demonstrated in the four 14q+ retinoblastoma lines using molecular techniques, and a probe mapping to the site implicated to have an activating role in lymphoma. These data suggest that there is a target for rearrangement at 14q32 but it is not the same sequence used in some Burkitt lymphomas. Two other breakpoints (2p24 and 8q24) coincided with the mapped position of cellular oncogenes, but also failed to show a molecular rearrangement with the oncogene probes. The breakpoints, 10q25 and 17p12, are constitutional fragile sites which may predispose these regions to act as acceptors of translocations in malignant cells. One line had double minute chromosomes, and was the only one of 16 (6%) tested with the N-myc probe which had an amplification. Different tumors from single patients with multifocal heritable retinoblastoma showed independent karyotype evolution. Unilateral non-heritable tumors exhibited a high level of karyotype stability throughout both in vivo and in vitro growth. The various common patterns of aneuploidy and translocations probably confer an early selective advantage to malignant cells, rather than induce malignant transformation.

Introduction

The germinal mutation predisposing to the dominantly inherited human tumor, retinoblastoma (RB), has been shown by family studies to be linked to the enzyme esterase D at chromosome band 13q14 (Sparkes et al. 1983). The occurrence of RB in patients with a congenital karyotypic deletion of this region (Yunis and Ramsay 1978) combined with analysis of age of occurrence (Knudson 1971) suggested that a deletion of gene function on chromosome 13 predisposed to RB tumors (discussed in Knudson 1983). Supporting evidence for this hypothesis was provided recently when it was shown that four out of six RB tumors from constitutional esterase D heterozygotes did not express both alleles in tumor cells (Godbout et al. 1983). Molecular studies of these and other tumors using chromosome 13 DNA restriction fragment length polymorphisms (RFLP), demonstrated that the majority of both heritable and non-heritable tumors become homozygous for RFLP over extensive regions of chromosome 13, always including the 13q14 band (Cavenee et al. 1983). Furthermore the mutant chromosome was always the one retained in tumors from patients with heritable RB (Cavanee et al. 1985). Similar homozygosities of chromosome 13 have been detected in osteogenic sarcomas occurring as second tumors several years after RB (Hansen et al. to be published). Despite some aneuploidy, the majority of RB tumors studied with RFLPs had two cytogenetically normal copies of chromosome 13. On this basis, it was proposed that novel chromosomal mechanisms such as mitotic recombination and/or duplication following loss, had generated homozygosity. Thus, molecular studies suggested that the RB mutation was recessive, and that the critical step in the transition to malignancy was the loss of the remaining normal allele at the 13q14 region. To explain the common aneuploidy of RB tumors it was suggested that cytogenetic abnormalities such as i(6p) might confer specific advantages during tumor development after primary mutations have occurred on chromosome 13 (Squire et al. 1984; Phillips and Gallie 1984).

We decided to analyze karyotypes of RB tumors in detail for two reasons. First, to determine the status of chromosome 13 as accurately as possible, checking for microdeletions or subtle rearrangements. Second, to determine if translocations were frequent at any preferential sites, particularly regions known to be associated with oncogenes, e.g., as for certain hematologic malignancies (Taub et al. 1984; Groffen et al. 1984). In our previous studies of RB (Squire et al. 1984; Gardner et al. 1982) we found aneuploidy predominantly in chromosomes 6 and 1, with chromosome 13 rarely affected. We have now karyotyped 27 different RB tumors, 12 of these in considerable detail. We report that only 3 out of 27 tumors appeared to be monosomic in the 13q14 region. Several tumors had common translocation breakpoints. Four of these translocations were at band 14q32, a site associated with the oncogene c-myc, however when examined with DNA probes,

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Table 1. Karyotype data from 27 RB tumors

Number	Type	Source[c]	Karyotype[a]	13[b] RFLPs	13q14 del	+6p	+1q
RB430[d]	B	PC	46,XY,t(1;?)(q44;?),−8,+der(8)t(1;8)(q22;q24),−10,+der(10)t(6;10)(p11;q25),t(17; ?)(p12;?)	Hom	no	yes	yes
RB267[d]	B	PMC	46,X ,+t(12;?)(q14;?),−16,+der(16)t(1;16)(q24;q24),−19,−Y,+mar	Hom	no	no	yes
RB429R[d]	B	PC	47,XX,+i(6p),−17,+der(17)t(1;17)(q11;p13)	Hom	no	yes	yes
RB429L	B	PM	48,XX,+1p−,−2,21p+,+mar1,+?mar2	−	no	no	yes
RB409[d]	B	PMC1	48,XY,+i(1q),t(5;?)(q35:?),+i(6p)		no	yes	yes
		C2	47,XY,+i(6p),−14,+der(14)t(2;14)(p11;q32),dir ins(14;13)(q24,q12,q32),ter rea(17;1)(p12;p11)	Hom	no	yes	yes
		C3	47,XY,+i(6p),−10,+der(10)t(1;10)(q12;p15),−14,+der(14)t(1;14)(q12;q32)		no	yes	yes
RB415[d]	B	PMC	65–67,XY,del(6)(q21),−17,+der(17)t(1;?;17)(1qter→1q12::?::17p12→cen→17qter)	−	no	no	yes
RB247a[d]	B	PMC	47,XY,del(1)(p32),+i(6p),t(9;?)(p22;?),t(16;?)(q24;?),−16,+mar1,+?mar2	−	no	yes	no
RB247c[d,f]	B	PMC	47,XY,+t(1;?)(p22;?),t(4;?)(p15;?),+t(6;?)(q16;?),t(9;?)(q31;?),t(9;?)(q31;?),+der(11)t(1;11)(q22;p15), 13p+,14p+,t(19;?)(q13;?),+mar3,+mar4,−14,−20,−22	Het	no	yes	yes
RB439	B	P	45–47,XY, +i(6p), +der(10)t(1;10)	−	no	yes	yes
RB448	B	P	47,XX,+i(6p)	−	no	yes	no
RB462	B	PC	46,XY,1p+,+der(12)t(6;12)(p11;q11),−16,+der(16)(1;16)(q11;q21),+mar,−6	M.R.	no	no	yes
RB181[e]	B	PMC	47,XY,t(1;X),t(2;?15),3q+,4p+,5q+,+i(6p),17p+	−	no	yes	yes
RB265[e]	B	PM	46,XX,t(1;15),t(1;16),dup(17)	−	no	no	yes
RB325[e]	B	P	46,X ,der(X)t(1;X)	−	no	no	yes
RB302a[e]	B	PC	47,XX,+i(6p),12p+,12q+,16q+	−	no	yes	no
RB302b	B	PC	?47,XX,dup(1q),+i(1q),5q+,7q+,−2,−1,+?mar	−	no	no	yes
RB355[d]	U	PMC	48,XY,−1,+der(1)t(1;1)(1qter→cen→1p36::1q12→1qter),+i(6p),t(17;?)(p13;?),+mar1,+dmin 50–100 (clone 7: all above markers plus 13p+, +mar2, +dmin 0–10)	Het	no	yes	yes
RB447	U	PMC	78–83,XXX,+i(6p),+i(6p),+ unidentified markers	−	no	yes	no
RB412[d]	U	PMC	47,XX,+i(6p),−10,+der(10)t(1;10)(q12;q26),−14,+der(14)t(1;14)(q12;q32),i(17q)	M.R.	no	yes	yes
RB405a	U	PC	47,XY,+t(1;?)(?q24;?),+i(6p),−10,+der(10)t(6;10)(p12;q25),13ps+,t(19;?)(q13;7),−20	Het	no	yes	yes
b[d]		PC	48,XY,+t(1;?)(?q24;?),−3,+der(3)t(3;6)(q26;q16),−6,+i(6p),−10,+der(10)t(6;10)(p12;q26),+13,13ps+, t(19;?)(q13;?)	−	no	yes	yes
RB414[d]	U	PMC	47,XY,−2,t(14;?)(q32;?),t(17;?)(p12;?),+mar1,+mar2 mar1 = der(2)t(1;1;2)(2qter→cen→2p25::1p13→1q22::1p13→1qter) mar2 = ?::1p13→1q22::1p13→1qter	−	no	no	yes
RB383[d]	U	PC	47,XY,t(1:?)(q43;?),+i(6p),t(7;?)(p22;?),−9,+der(9)t(1;9)(q23;p24),t(18;?)(p11;?),t(20;?)(q13;?)	−	no	yes	yes
RB445	U	P	45–46,XY,9q+,?−14,?22q+ (sl 46,XY,?17p+)	−	no	no	yes
RB455	U	P	44–47,XX,−13,+mar,?7p+	−	yes	no	no

RB386[e]	U	PM	47,XX,+i(6p)	–	no	yes	no
RB369	U	PC	E[e] 46,XY,5q+,7p+,–13,+mar1 mar1 = t(1q;?;7)	–	yes	no	yes
			A B C D } all lines have mar1				
RB381[c]	U	PM	46,XY,dup(1q),7q+,12q+,13q+,–13	–	yes	no	yes

[a] Abbreviated long form nomenclature (ISCN 1978) used to specify breakpoints in karyotypes of good quality; short form used for the remainder
[b] Chromosome-specific DNA restriction fragment length polymorphisms. Data previously published (Cavanee et al. 1983) and personal communication (Cavanee).
Hom = loss of heterozygous RFLPs, Het = retention of heterozygous RFLPs, M.R. = probably mitotic recombination, – = not tested
[c] Tumor samples were obtained from three sources; P, direct from patient; M, grown in nude mouse; C, grown in vitro.
The last letter indicates the source of tumor cells studied, the preceeding letters indicate the culture history of the tumor cells, for example,
PMC indicates tumor obtainecd from patient, grown in nude mouse, and passed to tissue culture
B, Bilateral and heritable RB; U, unilateral and probably non-heritable RB
[d] Karyotypes used in breakpoint analysis (shown in Fig. 3)
[e] These karyotypes have been published previously (Gardner et al. 1982)
[f] RB247c, this eye was irradiated prior to tumor removal

gene rearrangements were not detected. One tumor line had double-minute chromosomes and when analyzed for N-myc was found to have genomic amplification with 50–100 extra copies of this oncogene.

Materials and methods

RB tumors and cytogenetic studies

Cytogenetic studies were performed on 27 RB tumors derived from 24 individuals. None of the patients had any evidence of congenital abnormalities, constitutional chromosome abnormality, or microdeletion of chromosome 13. Sixteen patients with bilateral tumors or a family history were classified as heritable tumors, and the remaining unilateral tumors were considered non-heritable. Two or more independent tumors were obtained from each of three heritable cases. Tumor fragments were either analyzed immediately or cultured following the protocol described in the legend to Table 1. Chromosomes were prepared using methods described previously (Squire et al. 1984) and banded by trypsin-Giemsa methods (Seabright 1971). To insure that only chromosomes of adequate quality were analyzed, the number of discernible bands was estimated before attempting analysis. All karyotypes presented in this study were based on a minimum of ten fully analyzed metaphase photomicrographs. Twelve tumors derived from RB suspension cultures (Gallie et al. 1982) were analyzed in greater detail by a more rigorous selection of good quality metaphase cells for analysis.

Molecular studies

DNA was extracted from 5×10^7 cultured cells by standard techniques using phenol and chloroform/isoamyl alcohol, followed by ethanol precipitation (Maniatis et al. 1982); 10 µg were digested with restriction endonucleases (Boehringer-Mannheim GmbH) using conditions recommended by the supplier. Digested DNA was separated by gel electrophoresis in 0.8% agarose, and genomic blots were made onto nitrocellulose (Southern 1975). Recombinant plasmids pNb-1 ("N-myc", Schwab et al. 1983), pHuJH human immunoglobulin heavy chain J switch region (Adams et al. 1983), and pMC445 ("c-myc", Dalla Favera et al. 1982a) were radiolabeled with ^{32}p-dCTP by nick-translation (Rigby et al. 1977) to a specific activity of $5–9 \times 10^8$ cpm/µg and used to probe DNA bound to nitrocellulose. Washing of filters was carried out in $0.1 \times$ SSC ($1 \times$ SSC = 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0) and 0.2% sodium dodecyl sulfate (SDS) at between 55–60°C, and autoradiograms were developed after 24–48 h at −70°C.

Results

1. Summary of tumor karyotypes

All karyotypic data from each of the 27 tumors are presented in Table 1; some of the karyotypes have already been published in less detail (Gardner et al. 1982; Godbout et al. 1983; Squire et al. 1984). Direct chromosome analysis was possible from five tumor biopsies, but the quality of these karyotypes did not permit comprehensive analyses. The majority of the karyotypes and all the detailed analyses were carried out on tumor cells that had been grown in vitro or in immune deficient mice.

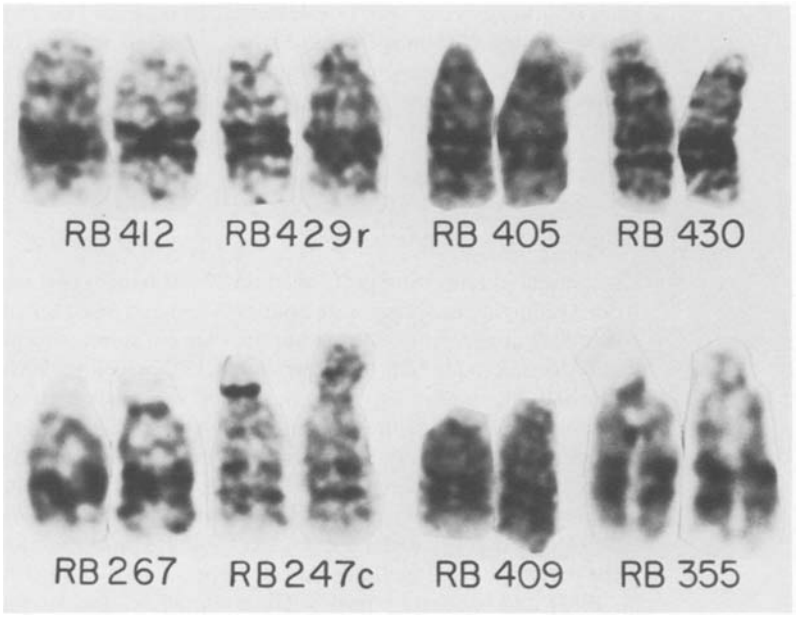

Fig. 1. Eight pairs of chromosome 13 from RB tumors stained using standard Giemsa-banding techniques showing no indication of 13q14 deletion

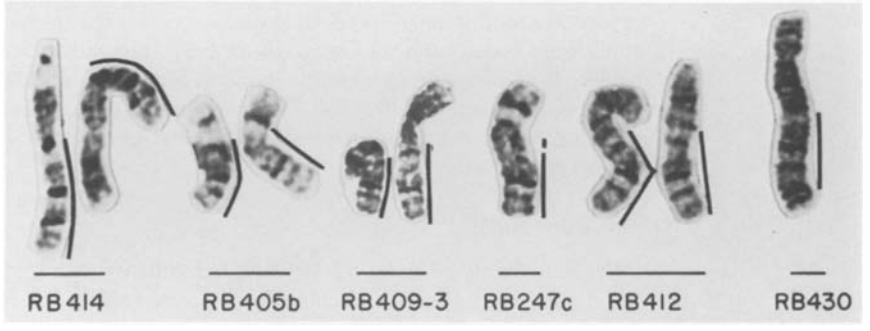

Fig. 2. Various translocations from six different RB tumors producing trisomy of the long arm of chromosome 1 (1q regions are indicated with *solid lines*)

Most tumors were near diploid or hyperdiploid with modes in the 46–48 range; two tumors had larger numbers of chromosomes with modes of 66 (RB415) and 80 (RB447), but count heterogeneity and nonspecific losses made analyses of these two tumors difficult. Three distinct sublines were detected in one heritable RB sample (RB409), with i(6p) as the only common marker. One unilateral tumor (RB445) had two sublines with no markers in common. One tumor line, RB355, had a large number of double-minute (DM) chromosomes (50–100 per spread) which were not apparent in the earliest cytogenetic analysis. The DM either developed during manipulation of the tumor, or the growth condition selected for cells with DM.

Chromosome 13 was aneuploid at band 13q14 in only 3/27 tumors: RB369, RB381, and RB455 (Fig. 1). However, extra copies of the short arm of chromosome 6 and the long arm of chromosome 1 (Fig. 2) were present in 15/27 (56%) and 21/27 (78%) tumors respectively; only one tumor, RB455, did not contain either of these two abnormalities. The +6p and +1q markers were present at similar frequencies in non-heritable and heritable RB: 6/11 (55%) non-heritable tumors were aneuploid for 6p (usually a distinctive short arm isochromosome), compared to 9/16 (56%) heritable tumors. Eight of eleven (73%) non-heritable tumors were trisomic for 1q compared to 14/16 (88%) heritable tumors. None of the karyotypes analyzed in detail (indicated in Table 1) had a detectable deletion or monosomy of chromosome 13 (Fig. 1). RB405b and RB409-C2 were trisomic for chromosome 13. Molecular studies reported previously (Cavanee et al. 1983) have shown that of eight tested tumors six have extensive regions of homozygosity in 13q (indicated in Table 1).

2. Cytogenetics of multifocal RB and karyotype evolution in unilateral tumors

Analysis of independent tumors from both eyes of three individuals with heritable multifocal RB (RB247, RB302, and RB249) (Table 2), indicated that abnormal karyotypes developed independently in each individual tumor. No marker was present in both tumors from the same RB patient; in fact, even the most characteristic marker of RB, i(6p), was absent from the RB429 left eye tumor but present in cells derived from the tumor of the right eye. These data clearly document the independent origin of different tumors in patients with heritable RB. In contrast are the tumor subsamples analyzed in four cases of unilateral RB (RB369, RB430, RB405, and RB355) (Table 2). RB405 for example, had a large single focus of tumor growth and separate isolates from the original tumor were grown following different culture protocols for several months before karyotyping (see RB405a and RB405b). All major chromosome abnormalities were present in both isolates which suggests that the tumors were capable of exhibiting a high level of karyotype stability under the strong selective pressure of in vitro growth. RB369 had five foci of

Table 2. Cytogenetics of RB multiple tumors and unilateral subsamples

Type[b]	Tumor	Culture[a]	Time[c]	Counts	Mode	Cells	Chromosome markers	Conclusions
U	RB369a	PC	6	40–47	47	11	> 90% cells have same large marker chromosome (mar1)	One tumor with multiple foci
	b	PC		[d]		15		
	c	PC		[d]		2		
	d	PC		[d]		15		
	e	PC		35–47	47	15		
U	RB430	PC	4	43–46	46	10	All examined cells have: 1q+, 8q+, 10q+, 17p+	Markers not induced by culture variables
		PM	5	39–46[d]	46	5		
		PM	4	33–46[d]	46	5		
U	RB405a	PMC	12	46–48	47	10	+1q+, +i(6p), 10q+, 19q+, −20	Slight differences after long periods of culture or when mass culture cloned
	b	PC	12	47–48	48	10	+1q+, 3q+, −6, +i(6p), 10q+, +13, 19q+	
U	RB355	PMC1	36	46–48	48	10	1p+,+i(6p), 17p+, +mar1,dmin 50–100	
		PMC1–7	24	46–48	49	10	1p+,+i(6p), −13,+13p+,17p+,+mar1.+mar2,dmin 0–10	
B	RB247a	PMC	11	39–47[d]	47	8	1p−, +i(6p), 9p+,16q+,+mar	Separate foci have completely different karyotypes
	[e]RB247c	PMC	24	42–46	46	10	+1p+,4p+,+?6p,9q−,11p+,13p+,14p+,19p+,+mar's	
B	RB302a	PC	12	45–48	47	17	+i(6p), 12p+, 12q+, 16q+	
	RB302b	PC	12	33–48	47	12	dup(1q), +i(1q), 5q+, 7q+, −2, −1,+?mar	
B	RB429L	PM	4	44–48	48	10	+1p−, −2, 21p+, +mar1, +?mar2	Tumors from different eyes have independent unique markers
	RB429R	PMC	4	38–48	47	14	+i(6p), +17p+	

[a] Tumor samples were obtained from three sources; P, direct from patient; M, grown in nude mouse; C, grown in vitro.
The last letter indicates the source of tumor cells studied, the preceeding letters indicate the culture history of the tumor cells, for example: PMC indicates tumor obtained from patient, grown in nude mouse, and passed to tissue culture

[b] B, Bilateral and heritable RB; U, unilateral and probably non-heritable RB

[c] Total number of months spent in culture before karyotyping

[d] High frequency of poor quality metaphases in these tumors

[e] Eye irradiated prior to tumor removal

tumor growth in one eye and was initially considered to be a de novo heritable case. However, the presence of a distinctive, large marker chromosome in each tumor focus makes this possibility unlikely; rather, the original tumor probably spread in the subretinal space to appear as separate retinal tumors.

RB355 was established as a cell culture line in August 1980; five months later a subclone RB355-7 was isolated. Detailed karyotypes prepared in 1983 showed a larger number (50–100) of double-minute chromosome in the parent line, RB355, and a much lower number (0–10) in the subclone RB355-7. The subclone contained all of the markers present in the parent and two new markers. Sakai et al. (1985) recently examined subcultures of RB355 and found that 45% of cells had no double-minute chromosomes, but had a homogeneously staining region (HSR) on chromosome 11. In our most recent examination of RB355, we were unable to detect an HSR or an altered frequency of double-minute chromosomes.

3. *Cytogenetic abnormalities and alterations of oncogenes*

One explanation that has been proposed to account for the characteristic chromosome markers of some malignancies is activation of transforming oncogenes by chromosomal rearrangements (Klein 1981). In our initial studies of RB karyotypes we noticed that particular breakpoints occurred frequently. It seemed possible that these translocations could indicate regions or genes of specific importance in RB; therefore, we decided to determine the overall distribution of all breakpoints. Because variability in chromosome band resolution in different tumors made it difficult to obtain reliable estimates of frequencies, we identified 12 tumors with high quality spreads permitting recognition of 300–400 bands in ten or more cells. Sixty-six breakpoints in the 12 tumors were recorded on a schematic idiogram and their locations were compared to the sites of all mapped cellular oncogenes (Fig. 3). Trisomy of the 1q23→1qter region generated by various translocations was very frequent (Figs. 2 and 4); 24/66 breakpoints occurred on chromosome 1, in most instances proximal to band 1q23. Three other sites in the genome appeared to be preferentially used as acceptor sites for translocations (Fig. 5): 10q25 (three tumors), 14q32 (three tumors), and 17p13 (five tumors). All incoming donor chromosome arms were different.

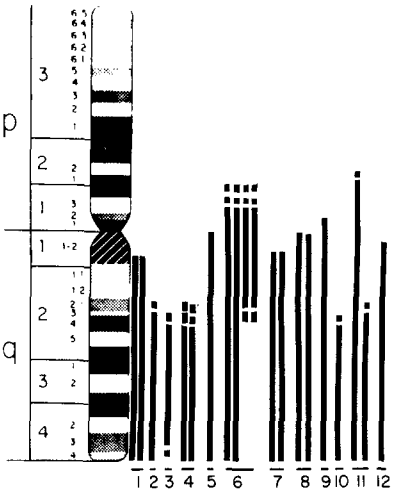

Fig. 4. Localization of the common trisomic regions *(solid lines)* of chromosome 1 using cytogenetic data from the 12 RB tumors analyzed in detail. Numbering from left to right: 1. RB412; 2. RB430; 3. RB383; 4. RB405B; 5. RB429R; 6. RB414; 7. RB409c3; 8. RB409c2; 9. RB409c1; 10. RB267; 11. RB247c; 12. RB415

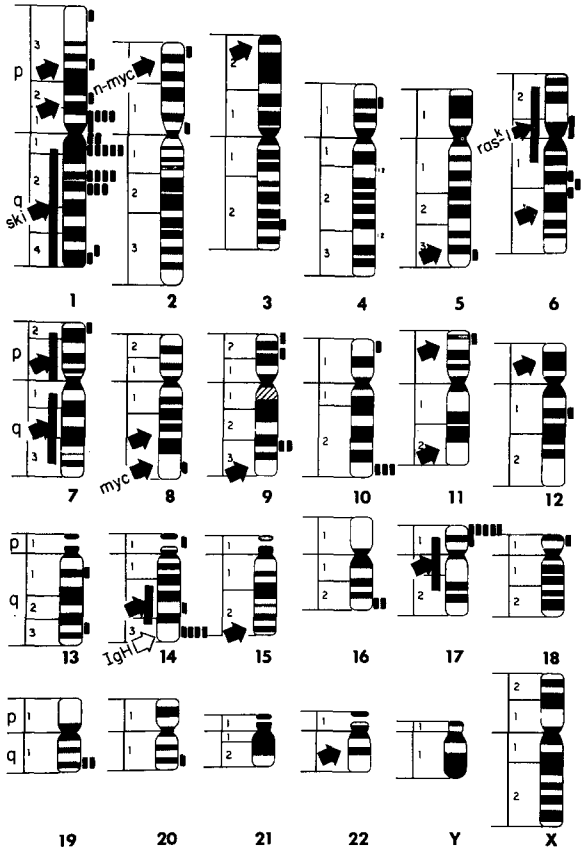

Fig. 3. Idiogram of human karyotype to indicate location of 66 breakpoints *(rectangles)* identified in 12 RB tumor lines and their proximity to the location of the mapped positions of the cellular oncogenes *(solid arrowheads)* and immunoglobulin heavy chain gene *(open arrow)*

One of the most intriguing observations of this study was the occurrence of translocations to the distal end of the long arm of chromosome 14. Of the tumors studied in detail three (RB412, RB414, and RB409) had different translocations affecting the same breakpoint, 14q32. In Burkitt lymphoma a balanced translocation between chromosome 8 and 14 places the incoming c-myc sequence near an immunoglobulin gene which may enhance the transcription of the translocated c-myc gene (Dalla-Favera et al. 1982b). About one third of Burkitt lymphomas with a t(8;14) translocation have molecular rearrangements detectable as a change in the restriction fragment size of the germ-line heavy chain class switch sequence (Adams et al. 1983). However, none of the RB lines, when tested with a probe for the switch region, had a similar rearrangement (Fig. 6). All RB lines with translocations involving 14q32 had unaffected 14kb bands characteristic of the germline configuration; any translocation must, therefore, occur at a distance greater than 14kb from the switch region. One line, RB430, had a translocation between chromosome 1 and 8 affecting band 8q24 which is the location of the c-myc oncogene. However, the c-myc DNA probe failed to detect any rearrangements in RB430 (Fig. 7a).

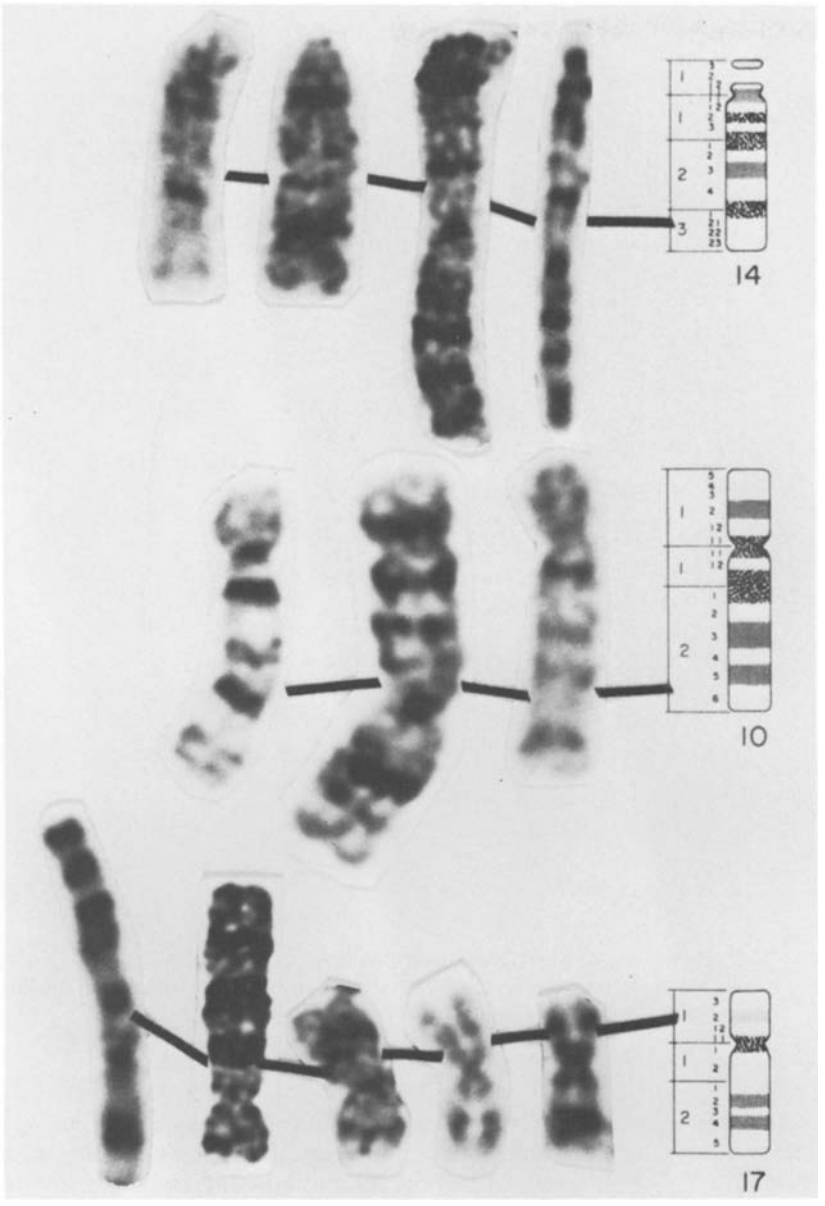

Fig. 5. Breakpoint determination in a detailed study of RB karyotypes. Numbering from left to right at 14q32 *(top)*: RB414; RB409-3; RB412; RB409-2, at 10q25 *(middle)*: RB405a; RB412; RB430, at 17p12 *(bottom)*: RB409-2; RB415; RB430; RB355; RB414. Details of donor chromosome translocation arms are described using ISCN nomenclature in Table 1

Oncogene amplification has been proposed as another activating mechanism (Bishop 1983). The oncogene N-myc (showing partial homology to c-myc), initially isolated from neuroblastoma, has often been found at high copy number when there is cytologic evidence of amplification, such as double minutes or homogeneously staining chromosome regions in neuroblastomas (Schwab et al. 1983). Recently N-myc was found to be amplified in some RB tumors (Lee et al. 1984; Schwab et al. 1984). The N-myc probe was used to test our RB cell lines for indications of gene amplification. Of the 16 tumors tested, evidence of gene amplification was found in two: RB355, a subclone RB355/7, and an established RB cell line, Y79-T (Reid et al. 1974) (Fig. 7b). Densitometric measurements of DNA dilutions of RB355 indicated fifty to one hundred-fold amplification. Lee et al. have reported N-myc amplification in 2 of 10 RB tumors, as well as Y79, whereas Schwab et al. did not find a case of amplification in three RB lines. The Kirsten ras oncogene (or pseudogene) is located on 6p, the oncogene ski is located on 1q (*arrowed* in Fig. 3). Involvement of these or other genes mapping to these chromosome arms in the aneuploidy for 6p and 1q observed in RB would have to involve activation by amplification at a much lower level than that occurring for N-myc.

Discussion

There is now solid molecular evidence that loss of genetic material including chromosome 13q14 is a primary mechanism leading to malignancy in RB (Cavanee et al. 1983). In that study most tumors had two normal appearing chromosome 13s, and the proposed mechanism of loss was mitotic recombination or loss and duplication leading to homozygosity of genes on the long arm of chromosome 13 including the mutant RB gene. However, frequent monosomy of chromosome 13 in RB tumor cells has been reported in some studies (Balaban et al. 1982; Benedict et al. 1983; Workman and Soukup 1984; Chaum et al. 1984) but not in others (Gardner et al. 1982;

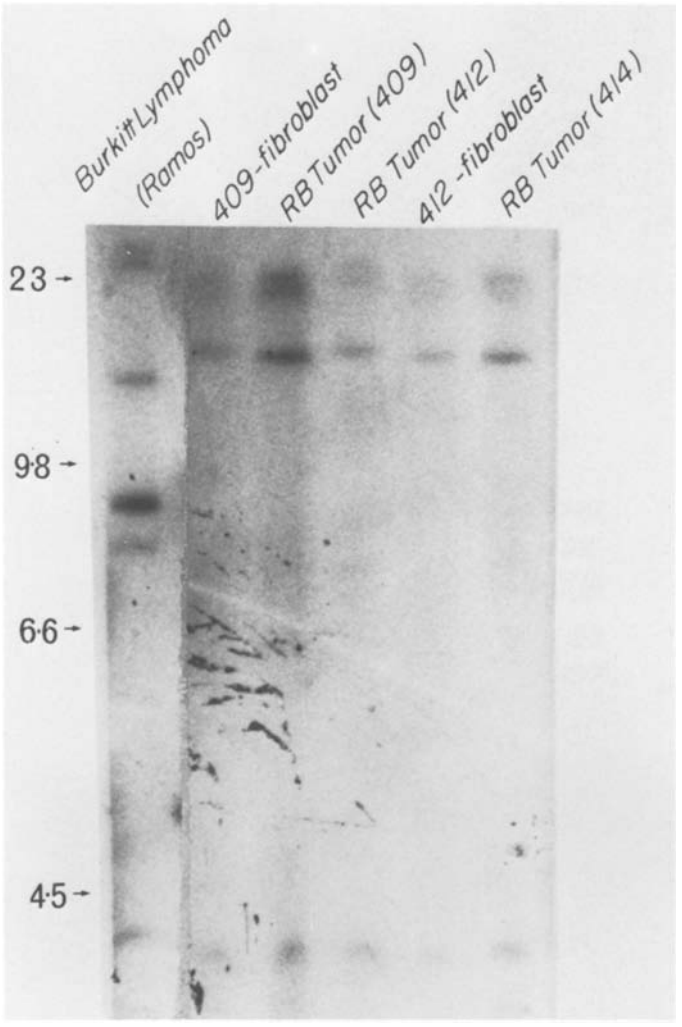

Fig. 6. Autoradiogram of Eco R1 digested DNA from cell lines with translocations at 14q32. DNA was probed with 8.0 kb human J region including Cμ switch

Kusnetsova et al. 1982; Squire et al. 1984). One of the initial aims of this survey was to resolve some of these inconsistencies by examining our best quality karyotypes for subtle rearrangements of chromosome 13. In this study, we found no evidence of frequent 13q14 deletions or consistent chromosome 13 abnormalities, but we did find consistent abormalities of other chromosome arms. Only three of the total of 27 tumors (11%) had indications of 13q14 monosomy. A pooled survey of all published RB tumors excluding our own (Balban et al. 1982; Benedict et al. 1983; Workman and Soukup 1984; Kusnetsova et al. 1982; Hossfeld et al. 1978; Chaum et al. 1984) shows an overall frequency of 13q14 monosomy (including small interstitial deletions of 13q14) in individuals with normal constitutional karyotypes was 14 of a total of 41 tumors (34%). Our data differ significantly from the total published data listed above ($\chi^2 = 4.61$, $0.05 > P > 0.02$). However this difference may be artefactual; we have found that metaphase preparations made directly from tumor biopsies are often difficult to interpret since they are frequently subject to differential band compaction, breakage, and random chromosome loss. Furthermore, the demonstration of mitotic recombination (Cavanee et al. 1983, 1985) using chromosome 13 restriction fragment length polymorphisms, indicates that loss of a normal homologue can occur without any detectable change in the karyotype.

Klein (1981) has proposed that the distinguishing chromosome abnormalities of any particular malignancy may inappropriately activate oncogenes whose expression is restricted to specific times during normal growth and differentiation. Further support for this theory has been provided by technical refinements applied to cancer cytogenetics (Yunis et al. 1978; Ikeuchi and Sasaki 1979), and the discovery that the breakpoints of the two most consistent chromosome markers of hematologic malignancies coincided with the positions of cellular oncogenes (reviewed in Astrin and Rothberg 1983). These developments suggested to us that useful information about the location and types of activation events at regions other than 13q14 in the karyotype of RB tumors may be provided by a detailed study of high quality banded chromosomes. In the 12 tumor lines giving the best chromosome spreads, we detected three distinct categories of cytogenetic abnormality on other chromosomes. First, there were frequent dosage changes of chromosome arms leading to 6p tetrasomy and 1q trisomy. Isochromosome 6p (i(6p)), an abnormality common only in RB, may be confering unique advantages to tumors of retinal origin (Squire et al. 1984). Trisomy of the region 1q23→1qter probably also provide a selective advantage but in a more general way, since it is the most commonly reported trisomy of cancer (Kovacs 1978; Brito-Babapulle and Atkin 1981). For reasons discussed previously,

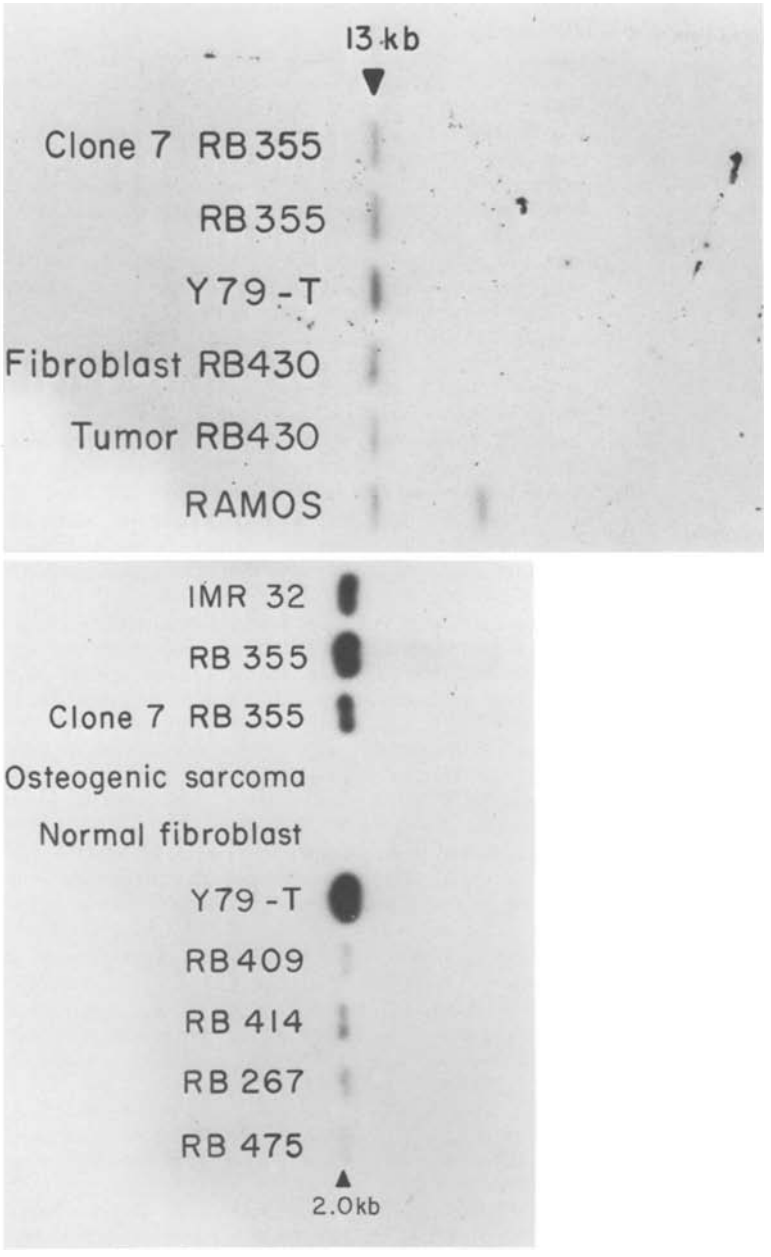

Fig. 7. a Autoradiogram of Eco R1 digested DNA from RB line and normal DNA. RB430 has a translocation at 8q24. DNA was probed with 3′ c-myc. **b** Autoradiogram of Eco R1 digested DNA from six RB lines, an osteogenic sarcoma with double minutes, and IMR32, a neuroblastoma line. DNA was probed with 1.0kb n-myc fragment

we believe that both of these chromosome changes are associated with progression rather than initiation of malignancy.

Second, there were frequent translocations occurring at three specific breakpoints in the genome: 14q32, 10q25, and 17p12. Our detailed study of selected metaphases provided the first indication that discrete cytogenetic abnormalities were detectable in RB. However, at present no oncogenes map to any of these three sites; the breakpoint 14q32 has been previously reported in RB as a 14q+ marker (Hossfield 1978) and appears to be associated with c-myc activation in Burkitt lymphoma (Erickson et al. 1983). Recently a sequence called bcl mapping to chromosome 11 in two B-cell leukemias with identical translocations at 14q32; t(11;14)(q13;q32), was found to have rearranged to the same immunoglobulin heavy chain region rearranging with c-myc in Burkitt lymphomas (Tsujimoto et al. 1984). Our failure to detect similar molecular changes accompanying the 14q32 translocations of RB makes it unlikely that this rearrangement leads to gene activation analogous to B-cell malignancies. Two of the breakpoints also involved in frequent translocations were 17p12 and 10q25; these regions are reported to be inducible constitutional fragile sites (Sutherland et al. 1984), and it has been suggested that the correlation of these fragile regions with cancer breakpoints may result from higher order differences of chromosome structure predisposing to chromosome translocation (Hecht and Sutherland 1984). Preferential translocation to fragile sites in RB tumors has also been found recently by Sasaki MS (personal communication). Two of our RB tumors had translocations close to the sites of known oncogenes; c-myc (RB430) and N-myc (RB414), but neither had a rearrangement when examined with molecular probes, suggesting that proximity to oncogenes in these lines may be fortuitous.

The final category of cytogenetic abnormality was cytologic amplification, observed as double-minute chromosomes in

RB 355. This was the only tumor of the 16 tested that had amplification of N-myc. N-myc is often amplified in neuroblastoma with 78% of cell lines (Schwab et al. 1984) and 38% of primary tumors having amplification (Brodeur et al. 1984). However N-myc appears to be less frequently amplified in RB, with only four of a total of 30 presently tested, showing amplification (this study; Sakai et al. 1985; Lee et al. 1984; Schwab et al. 1984). The significance of amplification of N-myc in RB is at present not clear; Lee et al. report that expression of this gene is elevated in two tumors that do not have gene amplification. Schwab et al. (1984) have not detected high levels of expression of N-myc in all neuroblastoma and RB tumors, and have suggested that amplification of N-myc is a late event, perhaps associated with tumor progression. The expression of cellular oncogenes in normal tissue at an early stage of differentiation is poorly characterized; however, it is possible that normal levels are perpetuated in tumors derived from embryonic target cells. Alternatively, increased expression of N-myc may confer a selective advantage with amplification being one of several ways that this increase is achieved.

These studies have provided insight into the different roles chromosomal alterations can play in the neoplastic process. Mutations of genes on chromosome 13 are probably the most important events in the etiology of RB tumors, and expression of the mutant phenotype (the tumor) is often achieved by the generation of hemizygosity or homozygosity at 13q14 using chromosomal mechanisms (Cavanee et al. 1983). The cytogenetic abnormalities we have described are probably late mutations, arising as a consequence of malignancy. Chromosome instability and karyotypic abnormality are hallmarks of malignancy. Abnormalities of cell division and chromosome separation may be expected to produce isochromosomes and generate translocations at an early stage of tumor growth. Both gross aneuploidy and specific translocations may be selectable by conferring immediate growth advantages to neoplastic cells. That multifocal tumors from individuals with multifocal, heritable RB acquire totally different chromosome abnormalities indicates that the RB germ-line mutation has little effect on the direction of tumor karyotype evolution or the type (+6p, or +1q) of aneuploidy generated. We suggest that these abnormalities of RB occur after the primary molecular mutations and chromosome rearrangements on chromosome 13 have taken place, and that they may be clues for future studies of the location of cellular genes important in the final expression of the RB tumor phenotype.

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