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Genetics

Molecular mechanisms of oncogenic mutations in tumors from patients with bilateral and unilateral retinoblastoma

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ABSTRACT **The *RB1* gene from 12 human retinoblastoma tumors has been analyzed exon-by-exon with the single-strand conformation polymorphism technique. Mutations were found in all tumors, and one-third of the tumors had independent mutations in both alleles neither of which were found in the germ line, confirming their true sporadic nature. In the remaining two-thirds of the tumors only one mutation was found, consistent with the loss-of-heterozygosity theory of tumorigenesis. Point mutations, the majority of which were C → T transitions, were the most common abnormality and usually resulted in the conversion of an arginine codon to a stop codon. Small deletions were the second most common abnormality and most often created a downstream stop codon as the result of a reading frameshift. Deletions and point mutations also affected splice junctions. Direct repeats were present at the breakpoint junctions in the majority of deletions, supporting a slipped-mispairing mechanism. Point mutations generally produced DNA sequences which resulted in perfect homology with endogenous sequences which lay within 14 bp.**

Retinoblastoma (RB) is an intraocular tumor with both sporadic and hereditary forms (1). In the familial form, linkage analysis has shown that a single gene (*RB1*) on chromosome region 13q14 is responsible for tumor predisposition (2–4). Individuals with bilateral, multifocal tumors are generally considered to carry germ-line mutations, whereas unilateral unifocal tumors are usually associated with sporadic RB (5, 6). Both copies of *RB1* must be inactivated for tumorigenesis to occur (7). The mechanisms leading to the loss of function usually involve duplication of an initial mutation with loss of the normal *RB1* gene (8). It has been assumed that, in those tumors where there is no loss of heterozygosity, two independent mutations occur, one in each allele (8, 9).

With the isolation and sequencing of *RB1* (10), it became possible to analyze the coding region of the gene. However, since the majority of tumors are treated successfully *in situ*, it is not always possible to analyze the mRNA for mutations. Even when tumor tissue is available, mRNA may not be produced (10) or there may be insufficient material available from which it can be isolated. Direct sequence analysis of the individual exons of *RB1* from genomic DNA was used successfully to identify mutations, although it proved a time-consuming process (11). More recently, techniques designed to identify mutations in *RB1* before sequencing have been employed (9, 12, 13). One of these, the single-strand conformation polymorphism (SSCP) technique, is highly sensitive, simple, and rapid and has been used to identify mutations in many different genes (13–18). SSCP is based on the sequence-dependent migration patterns of single-stranded DNA fragments as they are electrophoresed through a nondenaturing polyacrylamide gel. We previously applied this technique to the detection of constitutional, heterozygous mutations in patients with hereditary RB (13, 18). Here we describe a comprehensive study of the nature of mutations in *RB1* in a series of 12 RB tumors which gives further insight into the mechanisms leading to mutagenesis of this gene. Using this approach, we were able to analyze the patients' normal cells for germ-line mutations. At present, the only widely used method of identifying mutant gene carriers is through linkage analysis in families with a prior history of RB (4), but the ability to identify causative mutations in sporadic cases in the future will be invaluable in the clinical management of the disease.

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MATERIALS AND METHODS

PCR–SSCP Analysis of DNA. The primers used for PCR, their annealing temperatures, and the restriction enzymes used to reduce the fragment size were as described (13). Two additional primers were used in this study to amplify a 318-bp fragment containing exon 17, which improved the separation of mutant and normal DNA in SSCP gels: 20877 (5'-ACTTCCAAAAAAATACCTAGCTCAAG-3') and 23728 (5'-CATTCATGTGCATATGGCTAACAAA-3'). The annealing temperature was 55°C and *Dra* I digestion produced fragments 139 bp and 179 bp long. Two new primers, 10605 (5'-GTTTTTAGGTCAAGGGCTTAC-3') and 10606 (5'-ATCTCTAGCATATAGAGCCCCTT-3') were designed to analyze the poly(A) signal. When the 330-bp poly(A) region was digested with *Rsa* I, DNA fragments 102, 111, and 117 bp long were produced. SSCP analysis was as described (13), except that electrophoresis was at 60 W at 4°C, conditions that increased the sensitivity of mutation detection. The position of double-stranded DNA was determined by running an undenatured sample.

Direct Sequencing of PCR Products. DNA samples (19) from RB tumors which showed bandshifts in SSCP gels were sequenced directly from the PCR products by using the biotin/streptavidin-coated magnetic bead system (13). Sequencing of exon 17, however, required the inclusion of 7-deaza-dGTP in the reaction mixture to overcome the frequent termination of the reaction.

RESULTS

To investigate the nature of *RB1* mutations in RB tumors, an exon-by-exon analysis of DNA from samples from 12 unrelated individuals was performed. Exons 1–26, the coding region of exon 27, the promoter region, the poly(A) signal, and flanking intron sequences were analyzed in all cases. Nine tumors were from patients with unilateral RB and 3 were from patients with bilateral RB. A total of 16 different mutations detected by SSCP and confirmed by sequencing were distributed throughout the gene (Table 1). Nine (56%) were point mutations, 5 (31%) were deletions of 1–22 bp, 1 was a 1-bp insertion, and 1 was a TT → C change (Table 1). Although one of the point mutations was T → G and one was

Abbreviations: RB, retinoblastoma; SSCP, single-strand conformation polymorphism.

Table 1. Summary of mutations in the *RB1* gene from 12 RB tumors

Tumor	Type	Exon	Mutation Position	Mutation Change	Origin	Effect	LOH	Restriction enzyme site affected
GOS 13	U	13	1353	Δ22	S	Frameshift → stop	Yes	*Hin*fI
GOS 19*	U	1	179	C → T	S	Splicing ?	Yes	*Nsp*BII
GOS 45*	U	19	2076	Δ2	S	Frameshift → stop	No	
GOS 109	B	14	1508	T → G	N/A	M → R or new splice acceptor → frameshift → stop	Yes	
GOS 159	U	11	1210	C → T	S	R → stop	No	*Nla* III
		14	1501	C → T	S	R → stop		
GOS 197	B	4	638	Δ1	N/A	Splice donor → deletion of exon 4	No	
GOS 537	B	4	622	TT → C	S	Frameshift → stop	No	
GOS 551	U	24	2658	Δ4	S	Splice donor → frameshift → stop	No	
GOS 559	U	10	1096	C → T	N/A	R → stop	No	
		12	1173	C → T		Q → stop		*Bcl* I
GOS 561	U	4	538	Δ4	S	Frameshift → stop	No	
		17	1804	C → T	S	R → stop		*Taq* I
GOS 563	U	10	1105	G → T	S	E → stop	No	*Mbo* II
		17	1791	C → T	S	R → stop		
GOS 568	U	16	1576	◇1	S	Frameshift → stop	No	

The type of tumor is classified as U (unilateral) or B (bilateral). Nucleotide positions of the mutations in the cDNA are indicated. Point mutations are given for the coding strand: Δ, deletion; ◇, insertion. The number of base pairs deleted or inserted follows the appropriate symbol. The origin of the mutation is classified as G (germ line) or S (somatic); no germ line origin was demonstrated. In some cases, blood was not available (N/A). For affected amino acids, the one-letter code is used: R, arginine; M, methionine; E, glutamic acid; Q, glutamine. LOH, loss of heterozygosity.
*13q−.

G → T, 7 (78%) were C → T transitions. The majority of mutations are predicted to generate premature stop codons or affect splicing (Table 1).

At first, several exons were analyzed simultaneously by SSCP without enzyme digestion of the PCR product (Fig. 1).

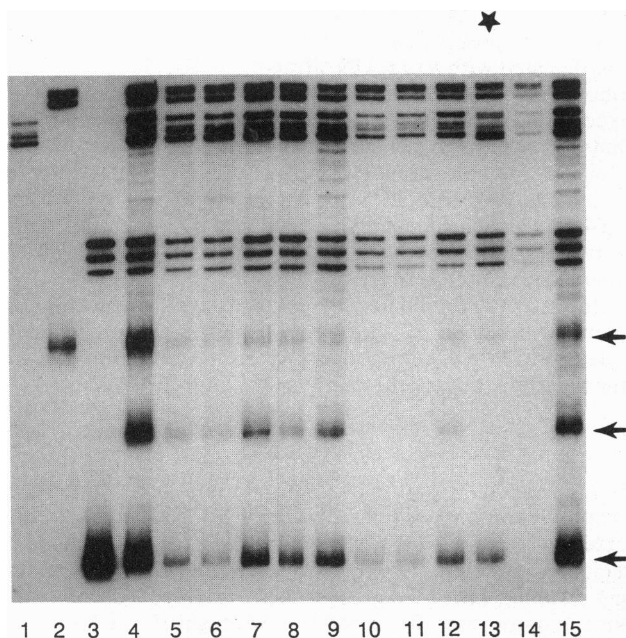

FIG. 1. SSCP gel illustrating simultaneous analysis (multiplex SSCP) of exons 4–6. Individual amplifications indicating the relative mobility of the PCR products are shown in lane 1 (exon 4), lane 2 (exon 5), and lane 3 (exon 6). Arrows indicate the position of the double-stranded DNA for each exon. Multiplex SSCP analysis of DNA from 12 different individuals is shown in lanes 4–15. In the DNA from tumor GOS 561 (lane 13, starred) an abnormal banding pattern for exon 4 is seen. The second band is missing and an additional lower band appears which was present together with the normal sequence.

However, maximal sensitivity was obtained when individual exons were amplified and these PCR products were digested with restriction enzymes (13). Representative SSCP gels which demonstrate band shifts corresponding to four different point mutations from exons 14 and 17 are shown in Fig. 2. In several tumors, both the normal and the mutant DNA sequence were observed. In tumors GOS 561, 563, 559, and 159, two different mutations were identified in separate exons (Table 1). The mutation in tumor GOS 45 appeared to be heterozygous, which is inconsistent with the fact that this patient carries a constitutional 13q14–22 deletion (20). In four tumors, despite the fact that there appears to be no loss of

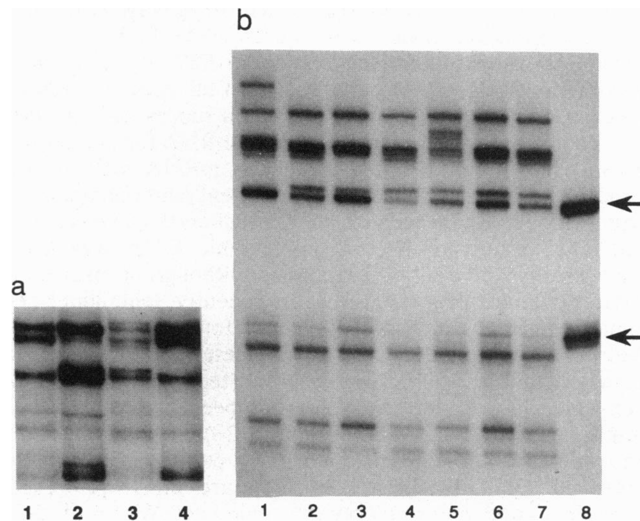

FIG. 2. (*a*) SSCP analysis of exon 14. The normal band pattern is given in lanes 1 and 4. In tumor GOS 109 (lane 2), two of the normal bands were noticeably faint or absent. In GOS 159 (lane 3), an extra set of bands was present. (*b*) SSCP analysis of exon 17 from seven tumors. Aberrant bands were present in GOS 563 (lane 1) and GOS 561 (lane 5). Double-stranded DNA is indicated by arrows.

heterozygosity, a second mutation has not been identified (Table 1).

Arginine (CGA) Codons Are Common Sites of Mutations. Seven (78%) of the nine point mutations were C → T transitions, all but one of which occurred in a CpG dinucleotide. Of the six CpG → TpG mutations, the only one which did not occur in a CGA arginine codon was in the third position of a GCC alanine codon. In this case, however, the next base in the coding sequence is a G, again creating a CpG doublet. Arginine is encoded by four different codons that contain CpG dinucleotides: CGT, CGC, CGA, and CGG. Of the 46 arginine residues in the RB protein the CGA codon is used 14 times in the *RB* gene (21) and 5 of these are the sites of C → T mutations in our series of tumors. The C → T transition generates a TGA stop codon. Exons 8, 14, 17, and 27 each contain two CGA codons. In our study (Table 1), both of the CGA codons in exon 17 and one of the CGA codons in exon 14 are mutated. Of the single CGA codons in exons 1, 10, 11, 15, 18, and 23, those in exons 10 and 11 were mutated (Table 1).

Somatic Frameshift Mutations Are Common. One-third of the abnormalities were deletions of 2–22 bp, representing the second most common form of mutation (Table 1). With the exception of tumor GOS 197, the deletions create a new reading frame and generate premature stop codons downstream (Table 1). The truncated proteins resulting from either a frameshift or an amino acid substitution would be expected to range from 133 to 837 amino acids in length.

Splice-Site Mutations. The mutations in tumors GOS 551 and GOS 197 affect the splice donor site, whereas, the mutation in tumor GOS 109, creates a new splice acceptor site. The 1-bp deletion in intron 4 in tumor GOS 197 removes the T of the invariant GT dinucleotide (Fig. 3*g*). The mutation in tumor GOS 551 does not affect the invariant dinucleotide, but the overall consensus splice donor site sequence is reduced (Fig. 3*d*). In tumor GOS 109, although the T → G mutation apparently converts a methionine to an arginine codon, a potential splice acceptor site might also be created, since an AG doublet followed by a G nucleotide is generated and is preceded by a pyrimidine-rich tract. Interestingly, a TACAAAC sequence, which bears strong homology to the mammalian branchpoint consensus sequence TNCT(A or G)AC (22) is also present 20–27 bp upstream from this mutation. If the surrounding sequence environment is appropriate and this splice acceptor site is used, a new reading frame would result in a stop codon at position 449.

Short Direct Repeats Flank Deletions and an Insertion. All of the deletions and the insertion were flanked by short direct repeats (Fig. 3), which have been postulated to participate in misalignment during DNA replication (see *Discussion*). The slipped-mispairing model is consistent with the generation of deletions observed in tumors GOS 45, GOS 13, GOS 561, and GOS 551 because one of the repeats, as well as the intervening sequence, is removed (Fig. 3). In some cases it is difficult to determine which nucleotide has been lost because of the repeated nature of the sequence. Although one possibility for each sequence is illustrated (Fig. 3), other interpretations are possible.

Point Mutations and Deletions Involve Quasi-Repeat Sequences. In tumors GOS 537 and GOS 197, where one complete repeat was not removed, a nearby sequence was observed which was identical to the mutant sequence. Similarly, a sequence which was at least 3–6 bp long and identical to the mutant sequence was found within 14 bp of eight of the point mutations (Fig. 4). In all of the C → T transitions the identical sequence was within <14 bp (Fig. 4).

The only mutation which would not affect protein structure is the C → T mutation in tumor GOS 19. Despite screening of all exons, the promoter, and the poly(A) signal from this

Tumor	Exon	Sequence
(a)	19	(645) ACC TC TC TTTCACTG
GOS 45		ACC TC TTTCACTG
(b)	13	tcctaaag (409) AAC .... AA TCCAAAAG AA (←14 bp→)
GOS 13		tcctaaag AA
(c)	4	(132) TTTA ACT T ACT AAAAG
GOS 561		TTTA ACT AAAAG
(d)	24	(840) GGG gt ga gt attttc
GOS 551		GGG gt attttc
(e)	16	AATG ACA ACA TTTTT
GOS 568		(478) AATG ACA A ACA TTTTT
(f)	4	(160) GCAC TC T TC AGC
GOS 537		GCAC TC C C AGC
(g)	4	(166) GAAAG gtaaa gtaaa ca
GOS 197		GAAAG g aaa gtaaa ca

FIG. 3. Direct repeat sequences at the sites of deletions and an insertion. Short direct repeats at the sites of five deletions (*a–d* and *g*), one insertion (*e*) and a TT → C mutation (*f*) are boxed. The repeat in exon 13 is imperfect and the nucleotide which differs is marked by an asterisk. The amino acid position is noted above the first triplet. Intron sequences are lowercase and exon sequences are uppercase. The normal sequence is shown in the upper line, and the mutant sequence in the lower line.

Exon	Tumor	Sequence	Substitution
1	GOS 19	GCC GCT	→ T
10	GOS 559	AAAC GATACGAAAT	→ T
10	GOS 563	AAGAAATTTATCTTAAAAATAAA	→ T
11	GOS 159	ACGAAAAAGTAACCTTGATG	→ T
17	GOS 563	AATGATAAAACATTTAGAACGAT	→ T
17	GOS 561	TGAACATCGAA	→ T
12	GOS 559	ATTCAGCAAGTGATCAA	→ T
14	GOS 159	TATTAC	→ T

FIG. 4. DNA sequences identical to the mutant sequence are adjacent to point mutations. The normal DNA sequence and the base substitution observed are described for eight point mutations. Sequences identical to the mutant sequence are underlined.

patient's tumor DNA with SSCP, the C → T mutation in exon 1 is the only observed change and this is in the third position of an alanine codon which does not change the amino acid.

Analysis of Constitutional DNA. The origin of the mutation was confirmed by screening lymphocyte DNA from the patient, wherever possible. Where the mutation affected a restriction enzyme site (Table 1), digestion of lymphocyte DNA with the appropriate enzyme was used to establish whether the mutation was constitutional. When a mutation did not affect a restriction enzyme site, the relevant exon from the patient's constitutional DNA was sequenced. In tumor GOS 19, the C → T transition apparently represents a silent mutation which was not present in the patient's lymphocytes. This individual carries a 13q14.3 subband deletion (20). Patient GOS 109 was bilaterally affected and had no known prior family history of RB. The T → G transversion identified in exon 14 is probably a new germ-line mutation, since loss of heterozygosity was observed, but we cannot rule out the possibility that the germ-line mutation may have been a large deletion. Blood cannot be obtained from this patient to confirm the origin of the mutation or the effect it will have on mRNA splicing.

DISCUSSION

We have used SSCP to identify mutations in 12 human RBs. Point mutations were the most common abnormality, the majority of the others being small deletions. Although 70% of RB tumors become homozygous for the causative mutation (8, 23, 24), only 33% of mutations in our series were obviously homozygous or hemizygous. In a further 33%, different mutations were found in both *RB1* alleles. In four tumors, despite screening of all 27 *RB1* exons, only heterozygous abnormalities were detected. In these cases it is possible that the normal DNA sequence was contributed by contaminating normal cells and the tumor had, in fact, become homozygous for the initial mutation. The same explanation is likely for the heterozygous mutation seen in tumor GOS 45 from a patient who carries a constitutional 13q deletion. Bilateral tumors are usually multifocal so it is possible that, in these cases, we are seeing contributions from different tumor foci with different second mutations. The mutation identified in tumor GOS 197, from a bilaterally affected individual, was heterozygous but was not detected in the patient's normal cells, so we presume there must be a second, unidentified mutation in this tumor which represents the germ-line mutation. It is possible that the mutation was either missed by SSCP or that it occurred in a different part of the gene. SSCP is very sensitive, in some instances detecting 100% of mutations (15). However, it failed to detect the germ-line mutation in tumor GOS 537; therefore it is entirely possible that some mutations will be missed.

Forty-four percent of all mutations, and 78% of all point mutations, in our series were C → T transitions, which is consistent with observations in other genes (25–29) and presumably due to the high mutability of 5-methylated cytosines in CpG dinucleotides (30). Although C → T mutations were reportedly more commonly germ-line mutations (11), we found a high proportion of somatic C → T transitions. This suggests that T–G mismatch repair is error-prone during both spermatogenesis (11) and mitosis.

In RB tumors, it appears that premature stop codons are usually required to inactivate the RB protein. Otherwise, a larger proportion of amino acid substitutions, caused by C → T mutations in CpG dinucleotides, would be observed. The C → T mutation in a CGA arginine codon is the only way a single base pair change in a CpG dinucleotide can convert an amino acid codon directly to a stop codon and is the most common finding. The CGA mutation in exon 11 seen in tumor GOS 159 has been reported in a different RB tumor (11); however, to our knowledge, mutation of either of the two CGA residues in exon 27 or the two CGA residues in exon 8 has not been reported. This may simply reflect the small number of tumors which we have studied so far.

The two-step misalignment model (31) proposes that a mutation arises when a sequence misaligns with a nearby highly homologous or quasi-repeat sequence. Thus, copying a quasi-repeat sequence during DNA replication establishes the mutation whereafter realignment returns the mutant sequence to its original position. In tumor GOS 197, for example, misalignment of a GTAAA sequence with the GAAA sequence located upstream could have resulted in excision of the nonhomologous T. Realignment then would produce the 1-bp deletion.

Although deamination of methylated CpG is often considered to be the mechanism for C → T transitions, our investigations suggest that local quasi-repeat sequences may also be important. In our series these repeats were all within 14 bp of the mutation. Hairpin loop structures (32), however, which might facilitate these misalignments, were not found. The fact that CpG dinucleotides are targeted implies that they are inherently unstable, since many other amino acid codons are capable of producing stop codons if a miscopying mechanism were to suffice. Thus, point mutations could arise through a misalignment mechanism except that, in this case, the quasi-repeat is the same length and a base substitution results. Sequences near quasi-repeats may be favored as sites of C → T mutations occurring via deamination of 5-methylcytosine because T–G mismatches may be stabilized by such a mechanism and protected from cellular DNA repair mechanisms. Misalignment may even promote deamination. Thus, the association of repeats and CpG sequences may not be random but may reflect a consistent mechanism. Such an association will, however, require the study of many more tumors.

Short direct repeats, 2–8 bp long, were found consistently at the sites of deletions in our study. Their presence at the breakpoint of short germ-line deletions has been documented (33) but here we report that short somatic deletions in *RB1* involve similar mechanisms. These deletions are thought to arise as a result of the "slipped-mispairing" mechanism (33–35). The size of the excision or insertion depends on the extent of mispairing. The 1-bp insertion in tumor GOS 568 is flanked by a 3-bp repeat but there is also a tandem repeat of a single base pair, A·T. The addition of 1 bp could result from a 1-bp misalignment of the tandem repeat and the looping out of the newly synthesized strand (31). Another A·T is then added resulting in an insertion. The 3-bp repeat may destabilize the DNA. It is interesting that deletion of the 3-bp repeat has been reported in a RB patient elsewhere (33). Although hairpin loop structures were suggested to bring the repeats together, we could not find any adjacent to any of our deletions that could facilitate misalignment.

A consensus sequence was noted in the vicinity of approximately half of the deletions seen in human genes (36). A highly homologous sequence was present at half of the six deletions described here. The same sequence is also similar to a common arrest site for DNA polymerase α. Krawczak and Cooper (36) suggested that arrest of DNA synthesis at the replication fork could facilitate the misalignment of repeat sequences and increase the chances of a deletion or insertion occurring. In tumor GOS 13 the mutation was located near a sequence identical to the (T or A)GAGG site where DNA polymerase α can arrest.

In our tumors, as in others (9, 12, 37–39), we have identified three potential splice-site mutations, two of which affect the splice donor site. These mutations would be predicted to generate premature stop codons, if exon-skipping occurs, by altering the translational reading frame. In the absence of a cryptic splice site, mutations of the splice donor site are usually associated with deletion of the preceding exon (37,

39–41). There are no obvious cryptic splice donor sites present in exon 4 or exon 24 or in the flanking 250-bp intron sequence in either case. It seems, therefore, that deletion of exon 4 in tumor GOS 197 and exon 24 in tumor GOS 551 is a more likely consequence. In tumor GOS 551, a new stop codon generated by the frameshift at position 838 would produce a truncated protein. *RB1* mutations that do not disturb the reading frame are exceptions in RB tumors (9, 12, 42). Although the deletion of exon 4 maintains the reading frame, it is not clear whether a stable protein would be produced. If so, this represents evidence that amino-terminal sequences are critical for RB protein function.

We have identified two mutations, (excluding tumor GOS 197) that apparently do not interfere with the translational reading frame. The effect of the mutation in exon 14 in tumor GOS 109 is not clear. It could convert a methionine to an arginine codon and maintain the reading frame, or it could create a new splice acceptor which, if used, would generate a premature stop codon. Unfortunately, insufficient tissue was available for mRNA analysis. Converting a methionine to an arginine residue is a nonconservative change and, if this is indeed the result, then this arginine residue must be critical for the function of the RB protein. Other examples of mutations which result in missense mutations in RB patients are rare (11, 18).

Although the C → T transition in tumor GOS 19 does not change the amino acid codon usage, it was not present in the patient's lymphocytes. Since patient GOS 19 carries a large constitutional 13q− deletion (20), the C → T transition must have occurred in the tumor in the homologous, nondeleted *RB1* gene. SSCP analysis has failed to identify another oncogenic mutation despite screening of all of the gene, and mRNA was again unavailable.

The importance of the local DNA sequence environment in mutagenesis is suggested by the DNA motifs which appear to predispose sequences to mutations. No apparent mutational hotspots occur but, from our studies, it would seem prudent to perform an initial screen of the exons containing the CGA arginine codons. The potential mechanisms underlying mutations in *RB1* suggest that the origin of many may be through DNA replication itself. It is not clear whether carcinogens and mutagens contribute to this process.

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