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# The specificity of UV-induced mutations at an endogenous locus in mammalian cells

**[pyrimidine dimer/(6-4) pyrimidine pyrimidone/DNA context]**

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ABSTRACT **We have used a rapid *in vivo* recombinational method to clone and completely sequence 34 UV-induced mutants at the adenine phosphoribosyltransferase (*APRT*) locus of Chinese hamster ovary cells. Among the mutants recovered, 26 were single base substitutions including 17 G·C → A·T transitions and a single A·T → G·C transition. Three of the 4 possible transversions accounted for the remaining 8 mutations. The G·C → T·A transversion was not recovered. Six tandem double or closely neighboring double-base substitutions, one double mutation consisting of a G·C → T·A transversion and an adjacent frameshift, as well as one single frameshift mutation were also recovered. UV-induced mutation appears to be targeted to dipyrimidine sites with only two exceptions. These include two double mutations where only one of the base substitutions occurred at a dipyrimidine site. The observed specificity of UV-light-induced mutations at the *APRT* locus is consistent with the argument that G·C → A·T transitions result primarily from the (6-4) pyrimidine pyrimidone lesion. A striking resemblance in the distribution of UV-induced mutants and a collection of 30 spontaneous mutants identified recently in our laboratory was noted. Both share a common strong site of multiple occurrence and a considerable degree of overlap with respect to site specificity. We speculate therefore that DNA context plays a significant role in mutation fixation in mammalian cells.**

The elucidation of mutagenic mechanisms is central to the understanding of carcinogenesis (1), heritable disease (2), and evolutionary processes (3). Information concerning the specificity of a particular mutagen may yield insights into the nature of the premutational lesions as well as the actual mutagenic pathways involved. Much important specificity data have been generated in recent years using elegant bacterial systems, especially those involving the *lacI* gene of *Escherichia coli* (4, 5). However, logistical problems have precluded such analyses in mammalian cells. To date no studies have described the DNA sequence alterations of a large collection of mammalian mutants at an endogenous locus. Although recent work with shuttle vectors has provided data on mutational specificity in mammalian cells (6–9), only by studying mutagenic specificity *in situ* can one be relatively certain that the DNA repair and replicative machinery of the cell is intact during mutagenesis.

UV light has been the most widely studied mutagen and is implicated as the major causative agent for cancer of the skin (10). In bacteria, many of the biochemical and genetic effects of UV light have been exhaustively documented and have led to a detailed understanding of the SOS response (11). Studies involving the disease xeroderma pigmentosum, an autosomal recessive disorder characterized by extreme UV sensitivity and predisposition to neoplasia of the skin, have led to a much greater understanding of excision repair processes in humans (12). However, the relative roles of the two major UV photoproducts, the (6-4) pyrimidine pyrimidone lesion and the cyclobutane dimer, remain controversial especially in mammalian cells. Evidence that the (6-4) lesion may play a dominant role in UV-induced transition mutagenesis has been obtained for bacteria (13–15) and λ phage (16). However, there is evidence that cyclobutane dimers are also capable of targeting mutation in both bacteria (17) and mammalian cells (18). The situation is further complicated by the fact that additional UV-induced lesions have been recently identified (19, 20).

Here we report on 34 UV-induced sequence alterations at an endogenous locus in Chinese hamster ovary (CHO) cells, obtained by using a rapid *in vivo* recombinational technique for cloning and sequencing mutant *APRT* alleles. The suitability of the adenine phosphoribosyltransferase (*APRT*) gene as a mutational target has been described elsewhere (21). The similarity of our UV-induced spectrum to others obtained in bacterial systems suggests a link between mutagenic mechanisms in prokaryotes and higher eukaryotes. Further insight is obtained by the striking resemblance of our UV-induced mutants to a collection of 30 spontaneous mutants recently cloned and sequenced in this laboratory (22).

## MATERIALS AND METHODS

**Maintenance of Cell Lines and Selection of Mutant Strains.** All $APRT^-$ mutants used in this study were derived from a clonal isolate of D422, an *APRT* hemizygote that was originally derived from a diploid CHO line auxotrophic for proline. Cells were routinely propagated on 100-mm Petri dishes (Nunc) in α minimal essential medium (GIBCO) supplemented with 5% fetal calf serum and 5% heat-inactivated horse serum (GIBCO). UV-induced mutants were collected by irradiating independent cultures containing $5 \times 10^5$ cells with a dose of 5 J/m$^2$ using a 254-nm UV source. After exposure, the medium was changed and a 5-day phenotypic expression period was allowed. Selections were performed by seeding two replicates of $5 \times 10^5$ cells per 100-mm Petri dish in medium containing 0.4 mM 8-azaadenine and 10% dialyzed fetal calf/inactivated horse serum. After 10–12 days, only one $APRT^-$ clone was picked per independently irradiated culture.

**Cloning and Sequencing of $APRT^-$ Mutants.** While the precise details of the cloning and sequencing methodologies are to be presented elsewhere (P. de Jong, A.J.G., J. de Boer, E.A.D., and B.W.G., unpublished results), the essential steps are outlined below. Mutant genomic DNA was isolated and size fractionated on 0.7% agarose to enrich for a 4.3-kilobase (kb) fragment containing the entire *APRT* gene (24). The resulting genomic fragments were then ligated to

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Abbreviation: APRT, adenine phosphoribosyltransferase.
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arms prepared from a defective λ vector pPDJ11 (*Aam/Bam*, a derivative of λ L47). A primary library was obtained and amplified on a host carrying the PiVX derivative pSDL12 (25), which carries the *supF* gene as well as 700 base pairs (bp) of DNA flanking the hamster *APRT* gene. High-frequency homologous recombination between the library phage containing the mutant *APRT* allele and pSDL12 results in the incorporation of the plasmid into the phage genome. Such recombinants can then be selected by plating on a nonsuppressor host. We have demonstrated that the entire *APRT* gene can be recovered on a single 5.7-kb *Bam*HI fragment in this manner. After recircularization, the fragment is then used to transform an F′ host strain. Since pSDL12 also contains a bacteriophage M13 origin of replication, single-stranded DNA for dideoxy sequencing (26) can be readily obtained after superinfection with M13. This *in vivo* enrichment procedure for the cloning of mammalian genes is described by Seed (25).

## RESULTS

**Mutation Induction and Cell Viability.** UV-induced 8-azaadenine-resistant mutants were collected by first seeding $5 \times 10^5$ cells on 100-mm dishes. Thirty-four such cultures were set up and each was irradiated with a dose of 5.0 J/m$^2$ from a 254-nm UV source. After a 5-day phenotypic expression period, the irradiated cells were reseeded in 8-azaadenine medium for the selection of APRT$^-$ clones. Only one 8-azaadenine-resistant colony was picked from each culture to ensure the independence of individual mutants. The UV dose used in this study was chosen because cytotoxicity was minimal, while induced mutagenesis was ≈45-fold over background (Table 1). This increase was sufficient to ensure that the spectrum obtained reflects that of UV-induced mutation.

**Characterization of *APRT*$^-$ Mutants.** The 8-azaadenine-resistant mutants were grown for 1 week in nonselective medium and then seeded in 8-azaadenine to confirm the stability of the mutant phenotype. High molecular weight DNA was then prepared from each mutant, restricted with *Bgl* II and *Hin*dIII, and analyzed by Southern blotting as described (21). None of the UV-induced mutants was characterized by an alteration in the size of the *APRT*-containing fragment, indicating that they did not result from large genomic rearrangements or deletions.

**DNA Sequence Analysis.** The 34 independent UV-induced mutants were cloned and single-stranded DNA was obtained. The entire coding sequence (540 bp) of each mutant was then determined. Among the collection (Table 2) were 26 single base substitutions. These included: 17 G·C → A·T and 1 A·T → G·C transition. The remaining 8 base substitutions included 4 G·C → C·G, 2 A·T → T·A, and 2 A·T → C·G transversions. No examples of G·C → T·A transversions were recovered. Four tandem double and 3 nontandem (closely neighboring) double mutations accounted for ≈20% of the UV-induced mutations (Table 2).

All of the single base substitutions and most of the double mutations occurred at dipyrimidine sites. One tandem and one nontandem double mutation each had one base substitution that occurred at a dipyrimidine site and an additional alteration opposite an isolated pyrimidine.

Table 1. UV-induced cytoxicity and mutagenesis

| Dose, J/m$^2$ | Relative survival | Mutation frequency, $\times 10^{-6}$ |
|---|---|---|
| 0 | 1.0 | 1.7 |
| 5.0 | 0.81 | 75 |

The location of UV-induced mutation within the dipyrimidine target was analyzed. Notably, every transition occurred at the 3′ side or within a run of pyrimidines. In contrast, of the eight transversions, half occurred unambiguously at the 5′ side of a potential dipyrimidine lesion. However, three of these did occur at one site. Of the remaining four transversions, two occurred within runs of pyrimidines and therefore are ambiguous, and two occurred on the 3′ side of a CT target.

**Multiple Occurrences or "Hot Spots".** Two sites within the *APRT* gene are characterized by multiple occurrences (hot spots). Three G·C → C·G transversions were recovered at nucleotide 1351, while five G·C → A·T transitions and one tandem double mutation occurred at position 241. The latter site lies within a 9-bp pyrimidine tract and was also identified as a hot spot in our spontaneous spectrum (22) (see *Discussion*).

## DISCUSSION

We have cloned and sequenced the entire coding region of 34 UV-induced APRT$^-$ mutants obtained in CHO cells. The sequence alterations recovered are likely responsible for the mutant phenotype, since in every case they result in either an amino acid substitution or the alteration of a splice acceptor site (Table 2). In addition, almost all of the UV-induced mutants analyzed were potentially targeted by photolesions occurring at dipyrimidine sites. The only exceptions were two double mutations where only one of the base substitutions occurred at a dipyrimidine target site.

G·C → A·T transitions predominate in the UV spectrum. This is not unexpected in light of previous work in both bacteria (15, 27, 28) and mammalian shuttle vectors (7–9) where a preferential induction of G·C → A·T transitions has also been noted. This has been explained by the preferential insertion of adenine opposite noncoding lesions such as has been demonstrated at apurinic sites in *E. coli* (29). This phenomenon may reflect properties of DNA polymerase (30) or the high dATP/dGTP ratios observed after UV irradiation of CHO cells (31). Certainly pool biases are able to affect mutation frequencies *in vivo* (32).

Recent controversy has centered around the relative contributions to mutagenesis of the two major UV-induced dipyrimidine photolesions, the (6-4) pyrimidine pyrimidone and the cyclobutane dimer. Brash and Haseltine (13) have suggested that the predominance of G·C → A·T transitions among nonsense mutations in the *lacI* gene is due to the mutagenic potential of the (6-4) lesion. More recently, Glickman *et al.* (14) have presented data indicating perhaps two-thirds or more of UV-induced mutation in *E. coli* is due to this photoproduct. Similar conclusions about the predominance of (6-4) photoproduct-induced transitions have been reached following the sequencing of several hundred *lacI* mutants in both Uvr$^+$ and UvrB$^-$ strains of *E. coli* (15). The specificity suggested for the (6-4) photolesions from these studies is G·C → A·T transitions at the 3′ side of potential dipyrimidine targets. Franklin *et al.* (33) have argued for the same specificity, based on physical chemical models that indicate the 5′ pyrimidine base of a (6-4) photoproduct retains its coding properties while the 3′ pyrimidone does not. The preferential insertion of adenine opposite the cytosine pyrimidone ring would then account for the predominance of G·C → A·T events. An examination of the G·C → A·T transitions in our collection is consistent with such a model. A preference for events at the 3′ side or the middle of a run of pyrimidines was observed. None occurred at the 5′ side of the potential lesion. In sharp contrast, four of eight transversions (three at the same site) occurred at the 5′ side of a dipyrimidine. Transversions at the 5′ side of dipyrimidine sequences may reflect mutations targeted by either cyclobutane dimers or some other yet to be characterized photo-

Table 2. Sequence analysis

| Mutant(s) | Position | Change | Amino acid | Target sequence (5′→3′) |
|---|---|---|---|---|
| | | Single base substitutions: Transitions | | |
| UV-26 | 58 | G·C→A·T | Pro→Ser | ACTTC C CCATC |
| UV-41 | 208 | G·C→A·T | Splicing | TCCCA G GGATA |
| UV-11 | 222 | G·C→A·T | Leu→Phe | CGCCC C TCCTG |
| UV-2, -4, -13, -35, -47 | 241 | G·C→A·T | Ser→Phe | CGCCT C CTTCC |
| UV-29 | 253 | G·C→A·T | Ser→Phe | AGCTT C CATCC |
| UV-54 | 1299 | G·C→A·T | Asp→Asn | GCCTA G ACTCC |
| UV-39 | 1303 | G·C→A·T | Ser→Phe | AGACT C CAGGG |
| UV-50 | 1309 | G·C→A·T | Gly→Glu | CAGGG G ATTCT |
| UV-27 | 1597 | G·C→A·T | Amber | AAATC C AGAAA |
| UV-1 | 1642 | G·C→A·T | Asp→Asn | TAGAT G ATCTC |
| UV-18, -32 | 1769 | G·C→A·T | Splicing | CCCCA G GAACC |
| UV-16 | 1770 | G·C→A·T | Gly→Glu | CCCAG G AACCA |
| UV-36 | 1854 | A·T→G·C | Leu→Pro | CTCAC T TAAGG |
| | | Single base substitutions: Transversions | | |
| UV-7 | 230 | G·C→C·G | Lys→Asn | CTGAA G GACCC |
| UV-51 | 244 | A·T→C·G | Phe→Cys | CTCCT T CCGAG |
| UV-34, -40, -56 | 1351 | G·C→C·G | Gly→Ala | CCTGG G CTGTG |
| UV-52 | 1381 | A·T→C·G | Leu→Arg | GAAGC T GCCAG |
| UV-44 | 1644 | A·T→T·A | Asp→Glu | GATGA T CTCCT |
| UV-33 | 1794 | A·T→T·A | Leu→Gln | TGAGC T GCTGG |
| | | Tandem double mutations | | |
| UV-10 | 59 | G·C→A·T | Pro→Leu | CTTCC CC ATCCC |
| | 60 | G·C→A·T | | |
| UV-24 | 213 | A·T→T·A | Ile→Tyr | GGGAT AT CTCGC |
| | 214 | A·T→T·A | | |
| UV-49 | 241 | G·C→A·T | Ser→Phe | CGCCT CC TTCCG |
| | 242 | G·C→A·T | | |
| UV-37 | 1887 | −T | Frameshift | ACCAT TC TTCTC |
| | 1888 | G·C→T·A | | |
| | | Nontandem double substitutions | | |
| UV-43 | 210 | G·C→A·T | Asp→Lys | CCAGG GAT ATCTC |
| | 212 | A·T→T·A | None | |
| UV-17 | 1301 | G·C→A·T | None | CTAGA CTC CAGGG |
| | 1303 | G·C→A·T | Ser→Phe | |
| UV-25 | 1376 | G·C→A·T | None | AGG G AAGCTG C CAG |
| | 1383 | G·C→T·A | Pro→Thr | |
| | | Other | | |
| UV-6 | 1853-54 | +T | Frameshift | CTCAC TT AAGGG |

product (19). We note that Protic-Sabljic *et al.* have shown that, for an extrachromosomal shuttle vector, cyclobutane dimers are associated with 3′ cytosine mutations and may play a significant role in transition mutagenesis in mammalian cells (18).

One approach to estimating the contribution of cyclobutane dimers to mutagenesis is to examine sites where (6-4) lesions rarely occur. TT and CT sequences represent sites at which (6-4) lesions are only detected after extremely high UV fluences (13). Among the 34 sequenced mutants, only 4 could have occurred at such sites: the single A·T → G·C transition (UV-36); the single base-pair frameshift (UV-6); and two transversions, an A·T → T·A (UV-33) and an A·T → C·G (UV-52). Two other events could have occurred at TT dipyrimidines, but the involvement of a TC lesion cannot be ruled out. These include an A·T → C·G transversion (UV-51) and a frameshift that occurred in tandem with a transversion (UV-37). The lack of mutation at thymine-thymine lesions may be accounted for if adenine is preferentially incorporated opposite UV photoproducts. However, the spectrum recovered here is consistent with observations made in *E. coli* (15, 28), which indicate that cyclobutane dimers as well as (6-4) lesions can produce mutation. Our data are also consistent with the recent suggestion by Schaaper *et al.* (15) that cyclobutane dimers are likely the cause of both transversions and frameshift mutations in *E. coli*. It may also be significant that the single A·T → G·C mutation was recovered at a site where the (6-4) photoproduct is not expected, raising the possibility that this event was targeted by another UV photoproduct, possibly the cyclobutane dimer.

The role for cyclobutane dimers in mutagenesis is perhaps most strongly associated with the production of tandem double mutations which have long been considered the hallmark of UV-induced mutagenesis (27). Indeed, in *E. coli* tandem double mutations have only been recovered following UV irradiation (4, 15), where they are considered the consequence of the noncoding nature of the cyclobutane dimer. The situation, however, is more complicated as 3 nontandem double mutations were also recovered in our study. Nontandem double mutations have been previously shown to be induced in *E. coli* by what appears to be a single event (34). Although nontandem double mutants comprise almost 10% of our collection, only 2 of 409 UV-induced *lacI* mutants were of this class (15). The prevalence of nontandem double events following UV irradiation in mammalian cells confirms an earlier report with the simian virus 40-driven shuttle vector pZ189, where 13 of 96 *supF* mutants sequenced were closely neighboring double events (8).

Two mechanisms may be proposed to account for such events. The simplest explanation is that damage distribution is nonrandom and that double mutations result from separate lesions. We consider this unlikely. For example, two double

events (UV-24 and UV-43) involve base substitutions at an isolated pyrimidine. Moreover, if lesions were so frequent, one would have to argue that multiple mutations would be scattered throughout the length of the target, and this is not the case.

A second mechanism suggested to account for tandem and nontandem mutations reflects the nature of the mechanistic processes that are associated with mutagenesis and DNA repair. For example, it has been proposed that the bypass of noncoding lesions such as cyclobutane dimers requires a reduction in the fidelity of DNA polymerase (35). Hence, to replicate past a dimer, the replication machinery may have a high risk of failing to insert subsequent bases accurately. An active reduction in proofreading stringency may not be required for bypass. The energy relay model of proofreading suggested by Hopfield (36) has the consequence of reducing replication accuracy with a loss of processivity. The relationship between accuracy and processivity has also been noted by Kunkel and Alexander (37).

Mutations are not recovered at random frequencies throughout the gene. In our collection of 34 mutants, 9 occurred at just two sites (see *Results*; Table 2). Of particular interest is position 241, which is the site of five G·C → A·T transitions and one tandem double event. The target sequence 5′ TCCTTCC 3′ (position 241 is boldface) is also present in the *supF* target carried on pZ189 (8). An examination of a mutational spectrum using this plasmid reveals this site to be a UV hot spot, as 9 of 96 mutations sequenced after passage through African green monkey kidney cells occurred there. In xeroderma pigmentosum cells, this site is also well represented, accounting for 13 of 61 sequenced mutations (38). The fact that the same sequence is a hot spot in both the shuttle vector and the endogenous gene may indicate that the processes involved in fixing mutation are similar.

The frequency at which different sites are represented in a mutational spectrum may be a function of both damage distribution and the competitive efficiencies of error-free and error-prone repair pathways. Brash *et al.* (39) have recently shown that in mammalian cells UV-induced mutational hot spots do not correlate with the frequency of photoproduct formation. Furthermore, repair processes may be of considerable importance since region-to-region and site-to-site variations in repair (40, 41) and the fidelity of DNA polymerase (42) have been observed. In light of this, we speculate that DNA context plays a major role in mutation fixation in mammalian cells. Site 241 also constitutes a mutational hot spot in the spontaneous spectrum accounting for 6 of the 30 mutations sequenced (22). It could therefore represent a site where fidelity is reduced, and bypass may occur here more frequently than at other sites. This may explain why position 241 occurs as a hot spot for both spontaneous and UV-induced mutation.

The significance of DNA context in UV mutagenesis might be further clarified by an analysis of the sites of multiple mutations. Intriguingly, five of the seven double mutations overlap with sites seen in the spontaneous spectrum (Fig. 1). In four of these five cases, the base substitutions are identical. Furthermore, of the nine sites of UV-induced single base substitutions shown in Fig. 1, five are also represented in the spontaneous spectrum. This degree of overlap would appear to lend credence to the argument for local context effects, since it may reflect a diminished accuracy of replication at these sites. While the overlap might reflect a paucity of available sites at which base substitutions can be recovered, this seems unlikely considering the diversity of sites recovered in other spectra in this gene (ref. 22; unpublished data).

This study has demonstrated the feasibility of examining mutational specificity at endogenous genes in mammalian cells. We note the similarity of the data obtained here with an

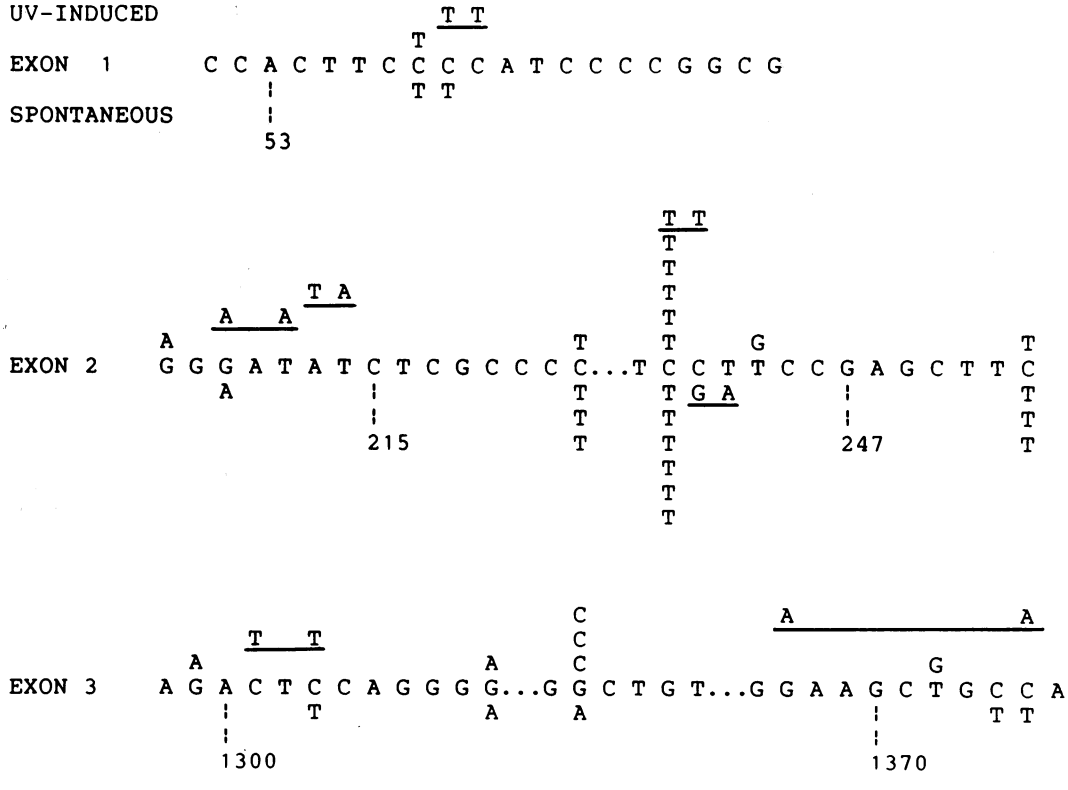


FIG. 1. Six regions of the *APRT* gene demonstrating the clustering of mutational events. DNA sequence is given in the 5′ → 3′ direction. Bases shown above the line indicate changes recovered after UV irradiation, while changes below the line indicate spontaneous events (22). The underlined bases indicate tandem and nontandem double mutations and dots represent positions where not all the sequence is given.

endogenous mammalian gene to that observed for UV-induced *lacI* mutants in *E. coli*. This may reflect common mechanisms of UV-induced mutagenesis in prokaryotes and higher eukaryotic organisms, and it is encouraging considering the vast database from bacterial test systems. Our data also confirm many observations made in shuttle vectors replicated in mammalian cells including the preference for G·C → A·T transitions in dipyrimidines and the induction of both tandem and nontandem mutations. Shuttle vectors only monitor direct mutagen–target interactions, however, and cannot be utilized to define other potentially important mutational parameters such as chromosomal aberrations (including nondisjunction, translocations, and inversions), mitotic recombination, and gene conversion. For example, recent studies have implicated a role for somatic recombination in oncogene activation in individuals heterozygous for the retinoblastoma gene (23). Such phenomena highlight the importance of developing the capability to study mutational specificity in endogenous cellular genes.

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