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Nucleotide Sequence Analysis of Human Hypoxanthine Phosphoribosyltransferase (*HPRT*) Gene Deletions

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We have determined the nucleotide sequences of 10 intragenic human *HPRT* gene deletion junctions isolated from thioguanine-resistant PSV811 Werner syndrome fibroblasts or from HL60 myeloid leukemia cells. Deletion junctions were located by fine structure blot hybridization mapping and then amplified with flanking oligonucleotide primer pairs for DNA sequence analysis. The junction region sequences from these 10 *HPRT* mutants contained 13 deletions ranging in size from 57 bp to 19.3 kb. Three DNA inversions of 711, 368, and 20 bp were associated with tandem deletions in two mutants. Each mutant contained the deletion of one or more *HPRT* exon, thus explaining the thioguanine-resistant cellular phenotype. Deletion junction and donor nucleotide sequence alignments suggest that all of these *HPRT* gene rearrangements were generated by the nonhomologous recombination of donor DNA duplexes that share little nucleotide sequence identity. This result is surprising, given the potential for homologous recombination between copies of repeated DNA sequences that constitute approximately a third of the human *HPRT* locus. No difference in deletion structure or complexity was observed between deletions isolated from Werner syndrome or from HL60 mutants. This suggests that the Werner syndrome deletion mutator uses deletion mutagenesis pathway(s) that are similar or identical to those used in other human somatic cells.

INTRODUCTION

Gene deletions play an important role in the genesis of heritable human disease. Prominent examples of human genetic diseases caused by germinal deletions include the Lesch–Nyhan syndrome (Yang *et al.*, 1984), familial hypercholesterolemia (Lehrman *et al.*, 1986, 1987; Hobbs *et al.*, 1987), hemophilia A and B (Furie and Furie, 1990), human growth hormone deficiency (Vnencak-Jones *et al.*, 1988), Duchenne and Becker muscular dystrophy (Liechti-Gallati *et al.*, 1989; Gilgenkrantz *et al.*, 1989), and a diverse range of hemoglobinopathies, including α- and β-thalassemia and the hereditary persistence of fetal hemoglobin (HPFH disease) (Nicholls *et al.*, 1985, 1987; Feingold and Forget, 1989; Henthorn *et al.*, 1990; reviewed in Kazazian, 1990). These human disease-associated germinal deletions range in size from 1 bp to >100 kb and genetically inactivate the affected allele.

Gene deletions also occur in human somatic cells *in vivo*. Gene deletion plays a prominent physiologic role in both the expression and diversification of immunoglobulin and T-cell receptor genes during B- and T-lymphocyte ontogeny (reviewed in Hedrick, 1989; Max, 1989). A role for somatic gene deletions in human disease pathogenesis is suggested by the reduction to homozygosity and allele loss observed at many genetic loci in human tumor cells. At least a portion of consistently affected loci are believed to encode tumor suppressor or lineage-specific differentiation genes (Ponder, 1988; Huebner *et al.*, 1989; Sager, 1989; Scrable *et al.*, 1990; Weinberg, 1991). One intriguing connection in this regard is the observation that neoplastic transformation or immortalization of several different human somatic cell lineages appears to be associated with an elevated frequency of gene deletions (see Fukuchi *et al.*, 1989).

The mutation pathways that generate human somatic and germinal deletions are not well understood. One way to begin to understand these pathways is to determine the structure of independent deletions in a defined gene at the nucleotide sequence level. We have used this approach to analyze the nucleotide sequence basis of deletion mutations in the human X-linked *HPRT* gene. Ten deletion mutants that were isolated as independent, spontaneous 6-thioguanine (TG)-resistant sublines from PSV-811 SV40-transformed Werner syndrome fibroblasts (Fukuchi *et al.*, 1989) or from HL60 human myeloid leukemia cells (Monnat, 1989) were used for these analyses. The deletion junction region was localized in the *HPRT* gene of each mutant by fine-structure Southern blot hybridization analysis, and then oligonucleotide primer pairs that flanked each junction were used to recover the junction region from mutant DNA for sequence analysis. Our aim was to determine and compare the nucleotide sequence basis of *HPRT* deletion mutagenesis in these two genetic backgrounds and

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thus determine whether the Werner syndrome deletion mutator we had previously identified (Fukuchi *et al.*, 1989) uses a qualitatively different pathway to generate deletions in human somatic cells.

MATERIALS AND METHODS

Cell culture. Werner syndrome and control SV40-transformed fibroblast cell lines (Fukuchi *et al.*, 1989) were grown in Dulbecco-modified Eagle medium supplemented with 6% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified 37°C, 5% CO_2/95% air incubator. HL60 cells (Collins *et al.*, 1977; Monnat, 1989) were grown in RPMI1640 medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified 37°C, 7% CO_2/93% air incubator. Cell lines were negative on repeated testing for the presence of *Mycoplasma* infection using a 4',6-diamidino-2-phenylindole (DAPI) fluorescence microscopy assay (after Chen, 1977).

Blot hybridization analyses. Southern blot hybridization was used to delineate *HPRT* gene structure in TG-resistant mutant cells. High-molecular-weight cellular DNA (10 μg), isolated by proteinase K/SDS solubilization and organic solvent extraction as previously described (Monnat, 1989), was first digested with 20 units of restriction endonuclease at 37°C (30° for *Pst*I) for 16–20 h in 40 μl of buffer recommended by the manufacturer. Digestions with two restriction endonucleases were performed sequentially with the adjustment of buffer composition prior to addition of the second endonuclease. The resulting restriction fragments were size-fractionated by agarose gel electrophoresis and transferred onto a nylon hybridization membrane (Nytran, Schleicher & Schuell or Hybond-N, Amersham) by capillary blotting as previously described (Monnat, 1989).

Hybridization probes consisted of the coding region or selected exons derived from a cloned human *HPRT* cDNA (Jolly *et al.*, 1983; Fukuchi *et al.*, 1989). Probes were radiolabeled with [^{32}P]dCTP (3000 Ci/mmol, Amersham) by random oligonucleotide-primed DNA synthesis (Feinberg and Vogelstein, 1983). Blot hybridization and wash conditions were as previously described (Monnat, 1989).

PCR amplification and DNA sequencing of deletion junctions. Oligonucleotide primers for deletion junction amplification and sequencing (Table 1) were synthesized by Operon Technologies (Alameda, CA), or by the Howard Hughes or the Molecular Pharmacology synthesis facilities at the University of Washington (Seattle, WA). Primers were purified by extracting once each with Tris-equilibrated phenol and with 24:1 (v/v) chloroform–isoamyl alcohol, followed by ethanol precipitation and resuspension in 10 m*M* Tris (pH 7.8)/1 m*M* EDTA buffer.

Amplification reactions contained 500 ng of mutant or control cellular DNA template, 200–400 ng of each primer, and 1–1.5 units of Amplitaq (Perkin-Elmer/Cetus) in 100 μl of 10 m*M* Tris (pH 8.3), 25 m*M* KCl, 2–3 m*M* $MgCl_2$, and 200 μ*M* dNTPs. Amplification reactions were performed in 0.65-ml microfuge tubes with a paraffin oil overlay in a Perkin-Elmer/Cetus thermal cycler. We typically performed 35 cycles of amplification, where each cycle consisted of a 94°C, 45 s–1 min denaturation step, and 42–58°C, 45 s–1 min primer annealing, and 72°C, 45 s–3.5 min extension steps. Amplification controls consisted of DNA from control cell lines and from a previously characterized HL60 mutant containing a complete deletion of the *HPRT* locus (HL60-3F9; Monnat, 1989).

Amplification products were analyzed by electrophoresis on 1% (w/v) agarose gels run in Tris–borate buffer. Small (<200 bp) fragments were analyzed on agarose gels supplemented with 1–3% (w/v) NuSieve GTG (FMC) agarose. Amplification products were purified by agarose gel electrophoresis followed by electroelution or by gel solubilization and binding to glass beads (GeneClean, Bio101) prior to sequence analysis.

Deletion junction and control *HPRT* fragments were sequenced by the dideoxy chain termination method using modified T7 DNA polymerase (Sequenase, USB). Sequencing primers (Table 1) were end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol, Amersham). Labeled primer (20 ng) was annealed to 200 ng of amplified DNA by heating primer-template mixes to 95–100°C for 5 min, then immediately placing reactions in a dry ice/ethanol bath. Chain termination reactions were performed by adding primed templates directly to ddNTP termination mixes. Products were run on 8% polyacrylamide/7 *M* urea gels that were dried prior to autoradiography as previously described (Monnat and Loeb, 1985). The nucleotide sequence of both strands of all junctions was determined, as were 50–100 bp of flanking 5' and 3' junction DNA sequence. DNA sequence data were compiled and analyzed using the University of Wisconsin Genetics Computer Group program package run on a VAX/VMS computer (Devereux *et al.*, 1984).

DNA sequence analyses. The 5' and 3' donor DNA sequences containing each deletion breakpoint were aligned with junction sequences and examined to determine how frequently donor breakpoints were found in or near sequence elements or motifs that have been implicated in chromosomal breakage, rearrangement, or deletion in mammalian cells (Table 2). We used the spatial equivalent of a failure-time analysis to determine whether the distribution of center-to-center distances from each donor breakpoint to the nearest copy of a sequence element or motif differed from the length distribution of that element in the human *HPRT* locus. The number of deletion donor breakpoints, n, was used to create n intervals each with probability $1/n$. The distribution of lengths for each probability interval was then calculated by solving

$$Y_i = -\ln(1 - P_i)\lambda,$$

for Y_i, the upper length boundary for interval i in basepairs. P_i is the cumulative probability of intervals $1 \rightarrow i$, and λ is the number of copies of the sequence element or motif in the human *HPRT* gene. For these analyses we used the *HPRT* exons 1–9 region (*HPRT* nucleotides 1704–41453; Edwards *et al.*, 1990) and assumed that both breakpoints and copies of each element were Poisson-distributed within the *HPRT* gene. The number of breakpoint center-to-element distances that fell within each length interval was tabulated, and a χ^2 value for the distribution as a whole was calculated by summing the squares of the observed number of occurrences within each length interval over all n intervals, then subtracting $n - 1$ to correct for degrees of freedom. We analyzed 25 donor sequences from 10 mutants and a subset of 12 donor sequences from 7 of the mutants that excluded double deletion and exon breakpoints.

We determined whether the frequency of deletion breakpoints in each *HPRT* intron differed from the frequency expected if the occurrence of breaks was directly proportional to intron length. For these analyses we used a set of 35 independent human *HPRT* deletion mutants, including the 10 described here, that contained a total of 51 breakpoints that have been unambiguously mapped to introns 1–8 (Fukuchi *et al.*, 1989; Monnat, 1989). Data for introns 7 and 8 were grouped to simplify the analysis. The statistical significance of differences in observed vs expected breakpoint frequencies were calculated for each intron ($df = 1$) and for the locus as a whole ($df = 6$) using a χ^2 test.

We also determined the degree of sequence identity and length of regions of identity between copies of *Alu* repeats contained in the *HPRT* exons 1–9 region using two different approaches. Separate files that contained each *Alu* repeat in a consistent (+) orientation (after Edwards *et al.*, 1990) were made then aligned and compared as a group with the GCG Pileup program (Devereux *et al.*, 1984). On the basis of the results of this comparison, 15 of the most closely related *Alu* repeat pairs were also aligned using the GCG Bestfit program to determine the length of regions of nucleotide sequence identity between repeat pairs.

RESULTS

Blot hybridization mapping of deletion junctions. Southern blot analyses with human *HPRT* probes con-

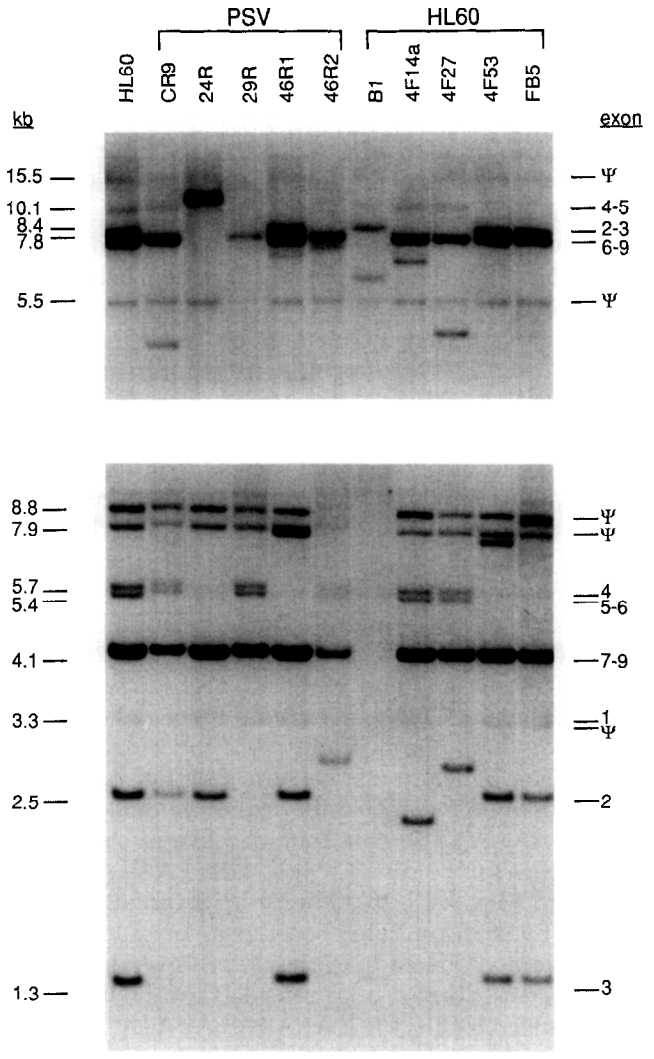

FIG. 1. Southern blot hybridization analyses of human *HPRT* gene deletions. DNA from control HL60 cells (lane 1) and from deletion mutants was digested with restriction endonuclease *Eco*RI (**top**) or *Pst*I (**bottom**) prior to blot hybridization analysis with a human *HPRT* cDNA coding region prone (Jolly *et al.*, 1983). Restriction fragment sizes are given in kilobases at the left, and the *HPRT* exon (e) or pseudogene (Ψ) content of each restriction fragment is given at the right. All mutant DNAs were missing one or more X-linked *HPRT* restriction fragment, and novel-sized restriction fragments were observed in all mutant DNAs with one or both restriction endonucleases with the exception of PSV811-29R. HL60-B1 DNA digested with *Pst*I was lost during sample preparation.

taining the complete cDNA coding region or selected exons (Jolly *et al.*, 1983; Fukuchi *et al.*, 1989) were used to determine *HPRT* gene structure and locate deletion junctions in each deletion mutant. Results obtained by digesting mutant and control DNAs with *Eco*RI or *Pst*I are shown in Fig. 1. Comparable blot hybridization analyses, performed after single or double digestion with the restriction endonucleases *Bam*HI, *Bgl*I, *Bgl*II, *Hin*dIII, *Kpn*I, *Puu*I, *Sac*I, and *Xba*I, revealed the loss of one or more restriction fragments and the appearance of novel-sized restriction fragments in each deletion mutant (additional data not shown). These blot hybridization data allowed us to construct internally consistent models of *HPRT* gene structure and to locate deletion junctions to within approximately 1 kb of *HPRT* sequence for all 10 mutants (Fig. 2).

Amplification and DNA sequence analysis of human HPRT deletion junctions. Oligonucleotide primer pairs (Table 1) flanking the predicted deletion breakpoints were used to amplify deletion junctions for sequence analysis. The identity of each amplified fragment was verified by restriction endonuclease mapping prior to DNA sequence analysis.

DNA sequence analyses of the 10 deletion junctions amplified from Werner syndrome and HL60 deletion mutants revealed three major junction types. Five "simple" deletion junctions had 1–5 bp of nucleotide sequence identity between donor sequences at the deletion junction and little or no donor nucleotide sequence identity 5′ or 3′ to the junction region (Fig. 3). These five deletions (PSV811-cR9, -24R, and -29R, and HL60-B1 and -4F27) ranged in size from 3.6 to 19.3 kb (Figs. 2 and 3).

Two deletions, PSV811-46R1 and HL60-4F53, contained additional "orphan" or "filler" nucleotides (Roth and Wilson, 1988; Henthorn *et al.*, 1990) at their junctions that were present in neither donor sequence. These two deletions encompassed 3.1 kb (PSV811-46R1) and 3.4 kb (HL60-4F53) of *HPRT* sequence and deleted exon 5 or 23 bp of exon 4, respectively. The deletion junction in PSV811-46R1 contained the insertion of four novel nucleotides (tatg in Fig. 4), that create a 6-bp direct repeat (TGTtatg) with a TGTTAT hexamer found 5 bp 3′ in junction sequence. This 4-bp insertion also creates two of a series of eight dispersed GTT direct repeats (GTtatgTT) contained in the 50 bp of sequence 3′ to the

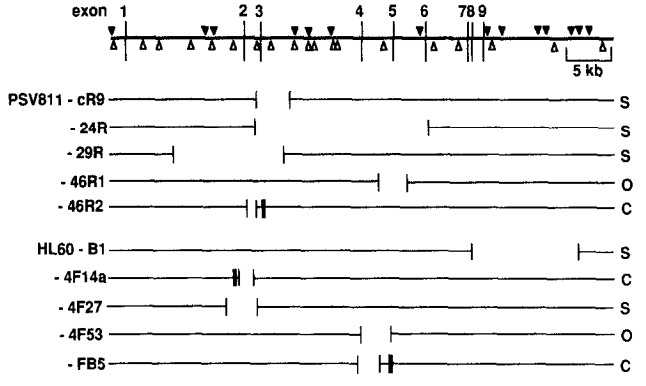

FIG. 2. Structural models of the *HPRT* gene in 10 human *HPRT* deletion mutants. The structure of the normal *HPRT* gene is represented by the solid line at the top of the figure on which the positions of exons (|) and restriction cleavage sites (▼, *Eco*RI; △, *Pst*I) are shown. The structure of the *HPRT* gene in each of 10 independent TG-resistant Werner syndrome (PSV811) or control (HL60) sublines, as determined by blot hybridization analyses, is shown below the normal gene. Deleted segments are shown as gaps bounded by vertical lines. Vertical lines within the gene sequence indicate the positions of second deletions with or without associated DNA inversions that were revealed by junction region DNA sequence analyses in three mutants (PSV811-46R2, HL60-4F14a, and HL60-FB5). The junction class for each mutant is given to the right. S, simple deletion junction; O, junction with "orphan" or novel nucleotides inserted; C, complex junctions with tandem deletions associated in two cases with DNA inversions.

TABLE 1

Oligonucleotide Primers Used for Human *HPRT* Deletion Analyses

Template[a]	(−) Primer/ *n*-mer[b]	(+) Primer/ *n*-mer[b]	Seq primer/ *n*-mer[b]
HL60-B1	39848/19	52324/22	—
HL60-FB5	25504/20	30655/22	29669(+)/19
	—	—	27426(+)/18
	—	—	30245(−)/21
HL60-4F14a	12760/24	16101/22	14179(−)/17
HL60-4F27	12760/24	306[d]/25	270(+)[d]/29
HL60-4F53	25504/20	31616/20	27798(−)/21
	—	—	31440(+)/18
PSV811-cR9	15710[c]/24	20172[c]/23	—
PSV811-24R	15710[c]/24	36545/21	35626(+)/19
PSV811-29R	7046[c]/21	20172[c]/23	19452(+)[c]/17
PSV811-46R1	29551/21	33642/22	33120(+)/19
	—	—	29792(−)/18
PSV811-46R2	220[d]/21	17269/21	15447(−)/19
	—	—	243(−)[d]/20
	—	—	16988(+)/24

[a] Templates were high-molecular-weight DNA isolated from the indicated mutant.

[b] Primer designations indicate the nucleotide position of the 3′-OH nucleotide of each primer in the human *HPRT* gene sequence (Edwards *et al.*, 1990). (+) primers hybridize to *HPRT* mRNA or to the message-equivalent DNA strand of the human *HPRT* gene, while (−) primers hybridize to the transcribed or "antisense" strand of the human *HPRT* gene. "*n*-mer" refers to the length of each primer in nucleotides. "Seq primers" refers to additional primers used for DNA sequencing.

[c] These primers contain a single nucleotide substitution when compared with the human *HPRT* gene sequence (Edwards *et al.*, 1990).

[d] Primer designations indicate the nucleotide position of the 3′-OH nucleotide of each primer in the human *HPRT* cDNA sequence (Jolly *et al.*, 1983).

deletion junction (Fig. 4). The 3′-most of these GTT repeats appears to have arisen from an A → TT substitution/insertion in the 3′ donor sequence (GAT to GTTT; Fig. 4). The deletion junction in HL60-4F53 contained an insertion of four thymidines that with junction nucleotides creates a direct 9-bp repeat (TGttttAAA) with an identical nonamer found 1 bp 3′ in the junction sequence (Fig. 4.).

DNA sequence analyses of the remaining three deletion junctions revealed closely spaced tandem deletions in association with novel nucleotide insertion (HL60-4F14a) or with 1 (PSV811-46R2) or 2 (HL60-FB5) DNA inversions (Fig. 5). The deletions in HL60-4F14a consist of a 57-bp deletion within the *Alu* repeat element 5′ to *HPRT* exon 2 and a 1.5-kb deletion that begins 48 bp 3′ to the first deletion in the same *Alu* element and extends 3′ into the next *HPRT Alu* repeat, deleting exon 2 (Fig. 6). The 57-bp 5′ deletion junction has an unusual structure that includes a 42-bp region of perfect donor nucleotide sequence identity immediately 5′ to the deletion junction, a 5-bp junction region containing three novel nucleotides (cGCat), and a 3′ region showing limited nucleotide sequence identity between donors. A G → T transversion was also observed 58 bp upstream of the 5′ boundary of this deletion junction. This substitution (shown as a t in Fig. 6) creates the fourth of four direct repeats ((GTTT)(GTtT)(GTTT)T(GTTT)) in an AT-rich DNA segment. The 1.5-kb 3′ deletion, despite having breakpoints in two *Alu* sequences, had but a single nucleotide of sequence identity between donor sequences at the junction (Fig. 6).

DNA sequence analysis of the deletion junction region contained in PSV811-46R2 revealed tandem deletions of 767 bp and 236 bp of *HPRT* DNA in association with a 368-bp inversion that includes 66 bp of *HPRT* exon 3. The remaining 117 bp of exon 3 are contained in the 5′ 767-bp deletion (Figs. 5, 6). Both deletion-inversion junctions in PSV811-46R2 were simple junctions with 3 bp (5′ junction) or 6 bp (3′ junction) of overlap between donor sequences and thus appear to have been generated by nonhomologous recombination. An AT-rich region ($(A)_7TGCATT(A)_5(T)_5$) was observed 5′ to the junction in the 5′ donor and junction sequences.

Nucleotide sequence analysis of the deletion junctions contained in HL60-FB5 revealed tandem deletions of 1990 bp (5′ deletion) and 97 bp (3′ deletion) of *HPRT* DNA that flank a 711-bp inversion. A second inversion of 20 bp was found immediately 3′ to the 97 bp deletion (Fig. 6). The 5′ deletion-inversion junction contains a 6-bp region of donor nucleotide sequence identity at the junction (AAAAATG) that included five of the six nucleotides that form a direct repeat with a AAAAAT hexamer found 7 bp 3′ (Fig. 6). The 20-bp inversion found 3′ to the 97-bp deletion has 3 bp of sequence identity between donors at the ends of the 20 bp inversion and is flanked by an inverted 5-bp repeat (GACTC). An AT-rich sequence block ($(A)_5(TAAA)_4ATACAAT$) was also observed in the 5′ donor sequence just 3′ of the breakpoint (Fig. 6).

The nucleotide sequences of the deletion junctions determined here have been deposited with the EMBL/GenBank/DDBJ Data Libraries under Accession Nos. M84530–M84540 and M84545. These deletion junction sequence data are cross-referenced to the human *HPRT* locus sequence determined by Edwards *et al.* (1990; Accession No. M26434).

Sequence analysis of deletion junction and donor sequences. Alignments of junction and donor sequences were scrutinized visually and machine searched to identify sequence elements (e.g., repeats and "simple-sequence" DNA) at or near junctions that might play a mechanistic role in deletion generation *in vivo*. Machine searches of junction and donor sequences were also performed to identify 33 sequence elements or motifs that have been associated with chromosome breakage, rearrangement, or deletion in eucaryotic cells (Table 2). Of these sequence elements, only the vertebrate topoisomerase I cleavage site CTY (Been *et al.*, 1984) appeared to be associated with donor breakage sites with a greater-than-chance frequency ($\chi^2 = 43$, $P \leq 0.01$ with $df = 24$ and $\chi^2 = 23$, $P \leq 0.025$ with $df = 11$). The association probabilities for CTC and CTT, the cleavage sites represented by CTY, were $P \leq 0.005$ for CTC ($\chi^2 = 47$

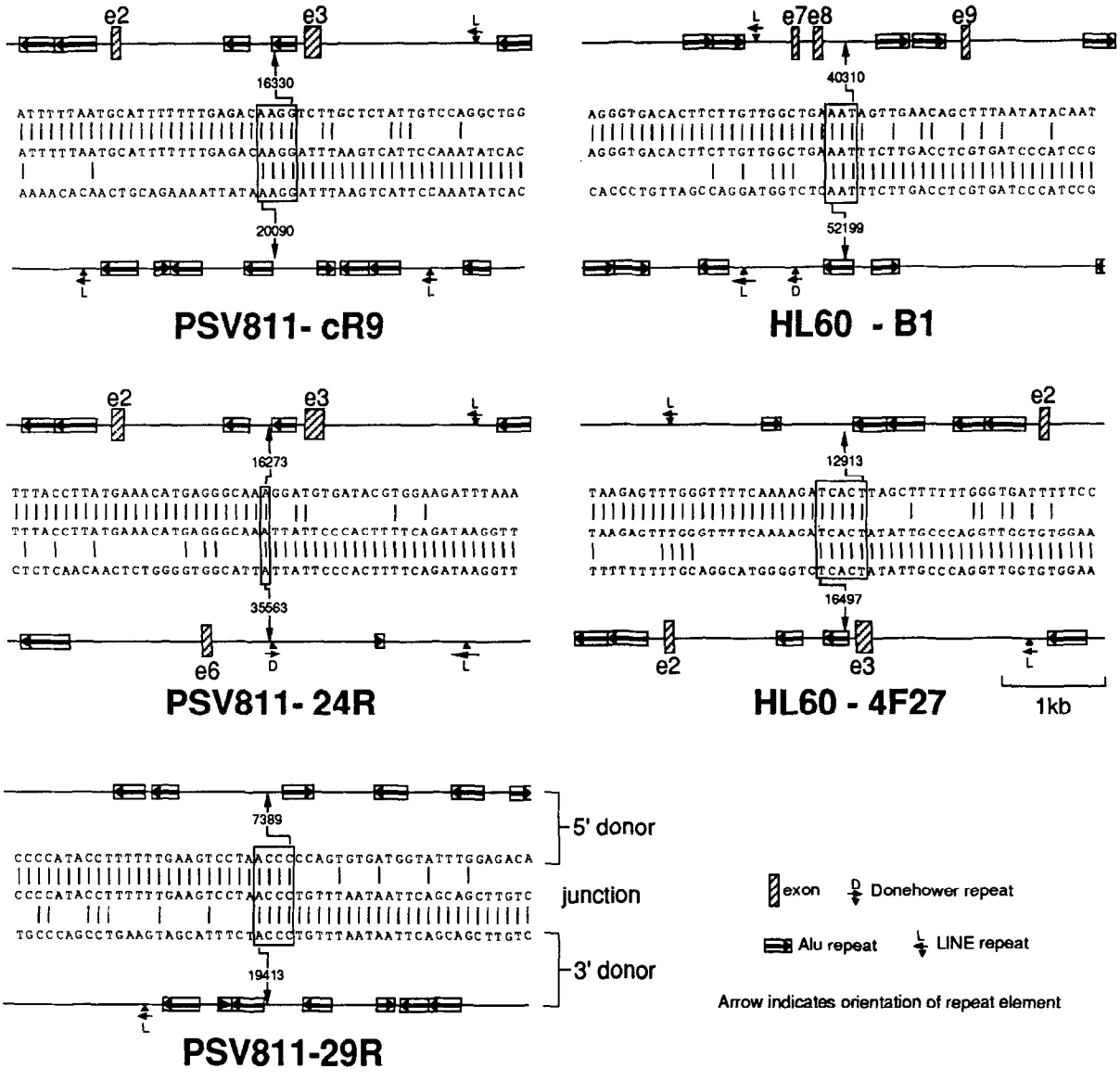

FIG. 3. Nucleotide sequence and structure of the simple *HPRT* gene deletion junctions. Junction sequences have been aligned with 5′ and 3′ donor nucleotide sequences for each junction. Five-kilobase segments of 5′ and 3′ donor *HPRT* gene sequence (horizontal solid lines) are shown above and below sequence data. Exons are indicated in these segments by hatched boxes, and the positions and orientations of repeated sequence elements are shown as boxed arrows (*Alu* repeats) or as labeled arrows (Donehower (D) and LINE (L) repeats). Donor nucleotide sequences are numbered after Edwards *et al.* (1990) to indicate the limit of sequence identity between junction and 5′ (**top**) or 3′ (**bottom**) donor sequences. Junction regions are indicated with solid-line boxes.

with $df = 24$) and $P \leq 0.1$ for CTT ($\chi^2 = 34$ with $df = 24$). Other topoisomerase I consensus cleavage sites had association probabilities that ranged from $P \leq 0.2$ (CAT with $df = 24$) to $P \leq 0.6$ (CAT or RAT) with $df = 11$. Topoisomerase CTY cleavage sites were found within 5 bp of deletion junctions in both DNA strands of 3, and one strand of 13, deletion junction donor duplexes. Only two of the deletions we analyzed, PSV811-29R and the 236-bp 3′ deletion in PSV811-46R2, did not contain a topoisomerase CTY cleavage site within 5 bp of the junction region in any donor strand (data not shown).

We determined whether donor breakpoints were clustered in the *HPRT* gene by comparing the frequency of expected with observed deletion breakpoints in each *HPRT* intron. There was a greater than expected number of breakpoints in introns 2 and 4 (16 or 10 versus the expected 7 or 5.5 in the $n = 51$ and $n = 40$ data sets, respectively) and a lower than expected number of breakpoints in intron 3 (8 or 6 versus 14.5 or 11.3 in the $n = 51$ and $n = 40$ data sets, respectively). However, these departures from expectation reached statistical significance at a $P \leq 0.05$ level only in the larger ($n = 51$) data set including double deletions.

We also determined the degree of nucleotide sequence identity between different *Alu* repeats found in the human *HPRT* locus. These alignments revealed two major blocks of sequence similarity among *Alu* copies of approximately 130 bp and 210 bp that correspond to the two *Alu* element "arms" (Kariya *et al.*, 1987; Deininger, 1989). Few segments of sequence identity between *Alu* pairs exceeded 30 bp, however (search results not shown).

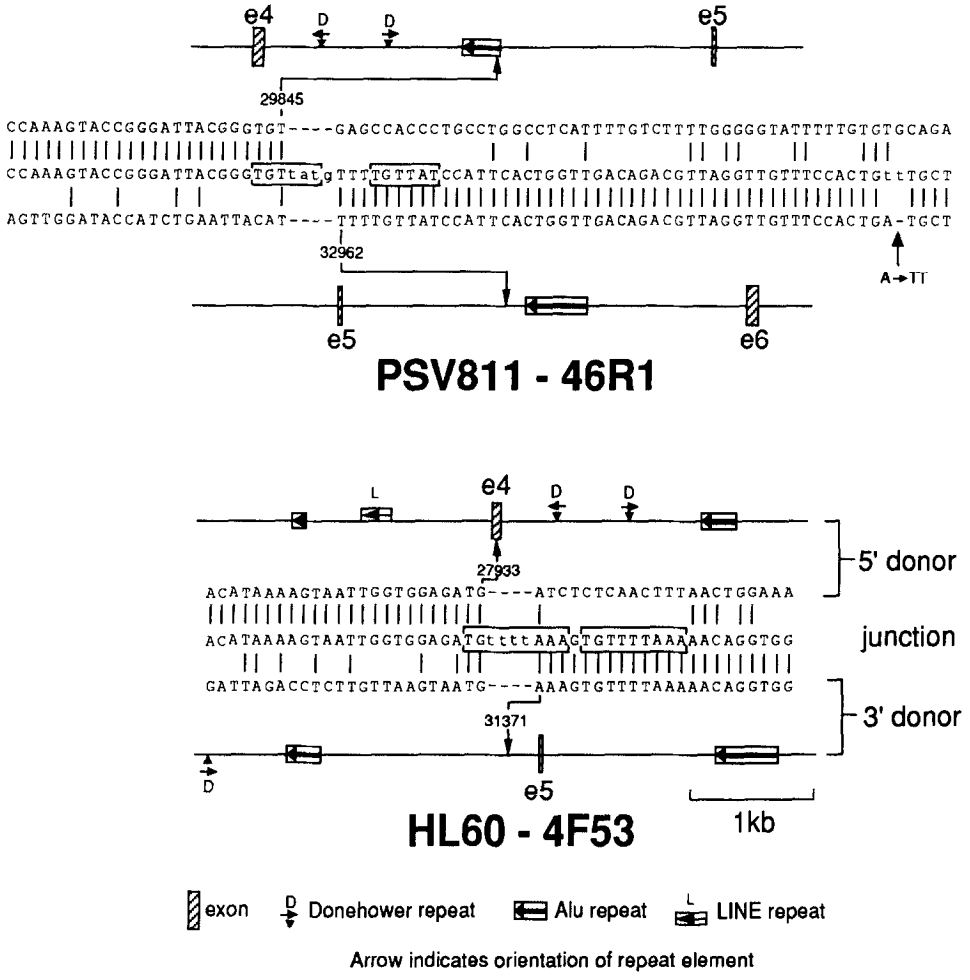

FIG. 4. Nucleotide sequence and structure of *HPRT* gene deletion junctions containing nucleotide insertions. Junction sequences have been aligned with 5′ and 3′ donor nucleotide sequences for each junction. Five-kilobase segments of 5′ and 3′ donor *HPRT* gene sequence (horizontal solid lines) are shown above and below sequence data. Exons are indicated in these segments by hatched boxes, and the positions and orientations of repeated sequence elements are shown as boxed arrows (*Alu* repeats) or as labeled arrows (Donehower (D) and LINE (L) repeats). Donor nucleotide sequences are numbered after Edwards *et al.* (1990) to indicate the limit of sequence identity between junction and 5′ (**top**) or 3′ (**bottom**) donor sequences. Novel or "orphan" nucleotides inserted at deletion junctions are shown in lowercase. Direct repeats created by these insertions are enclosed in horizontal brackets. An A → TT substitution in the PSV811-46R1 junction sequence is shown to the right of the junction below the 3′ donor sequence.

DISCUSSION

We have determined the structure of 10 human *HPRT* deletion junctions at the nucleotide sequence level. These deletions were originally isolated as independent, spontaneous 6-thioguanine-resistant mutants from PSV811 Werner syndrome cells or from HL60 human

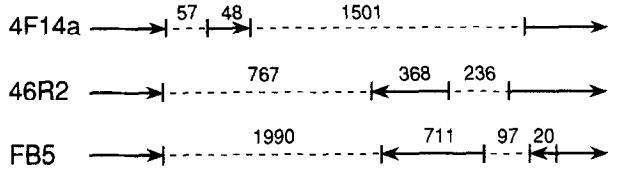

FIG. 5. Structural models of three complex human *HPRT* gene deletions. Deleted segments of the *HPRT* gene in three independent deletion mutants are shown as dashed lines. Remaining segments of the *HPRT* gene in each mutant are shown as right arrows (→) or, if inverted, as left arrows (←). Vertical lines indicate deletion or deletion-inversion junctions. The length of deleted or inverted segments are given in basepairs above each segment. N.B.: segment lengths are not drawn to scale.

myeloid leukemia cells (Fukuchi *et al.*, 1989; Monnat, 1989). Werner syndrome (WS) is a rare, autosomal recessive human genetic disease characterized by the appearance, in young adults, of features reminiscent of premature aging (Epstein *et al.*, 1966). Cells and cell lines from WS patients demonstrate chromosomal instability, and WS patients have an increased risk of developing malignancies (Salk, 1982; Sato *et al.*, 1988). We have recently identified a spontaneous deletion mutator phenotype in cell lines derived from WS patients (Fukuchi *et al.*, 1989) and an eightfold elevation in the frequency of TG-resistant T-lymphocytes in peripheral blood samples from WS patients (Fukuchi *et al.*, 1990). HL60 cells, in contrast, have a low spontaneous mutation rate at the *HPRT* locus, and a wide spectrum of molecular abnormalities have been identified in the *HPRT* gene of *HPRT*-deficient, TG-resistant HL60 cells (Monnat, 1989).

Sequence analyses of the deletion junctions amplified from 10 independent mutants revealed 13 separate dele-

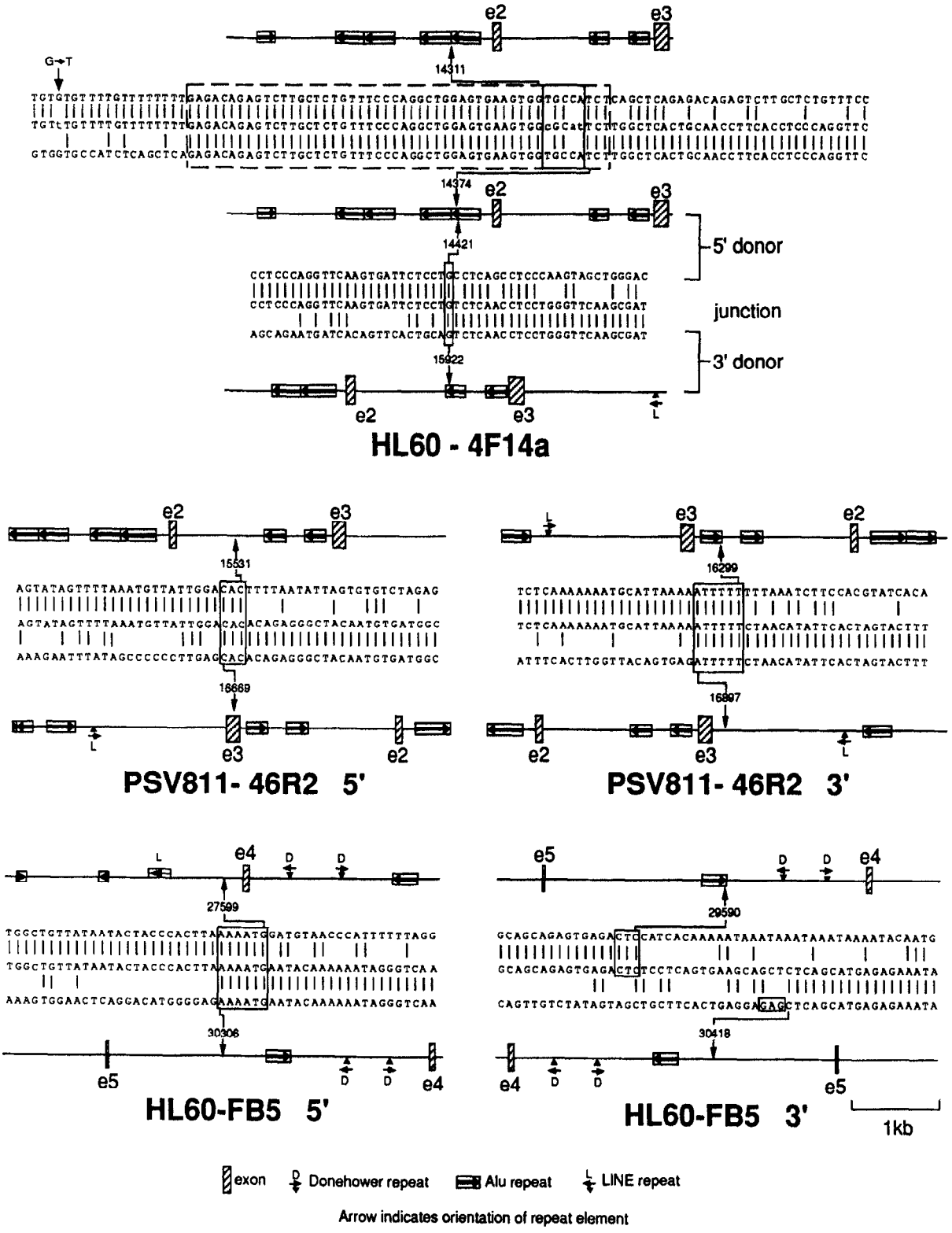

FIG. 6. Nucleotide sequence of complex *HPRT* gene deletion junctions. Junction sequences have been aligned with 5′ and 3′ donor nucleotide sequences for each junction. Five-kilobase segments of 5′ and 3′ donor *HPRT* gene sequence (horizontal solid lines) are shown above and below sequence data. Exons are indicated in these segments by hatched boxes, and the positions and orientations of repeated sequence elements are shown as boxed arrows (*Alu* repeats) or as labeled arrows (Donehower (D) and LINE (L) repeats). Donor nucleotide sequences are numbered after Edwards *et al.* (1990) to indicate the limit of sequence identity between junction and 5′ (**top**) or 3′ (**bottom**) donor sequences. Junction regions are indicated with solid-line boxes. For mutant HL60-4F14a, dashed line boxes enclose 42-bp 5′ and 3-bp 3′ regions of nucleotide sequence identity between donor sequences at the 5′ deletion junction and nucleotide substitutions in the junction are shown in lowercase. A G → T substitution in HL60-4F14a is indicated above the 5′ donor sequence. In the FB5 3′ junction, solid-line boxes indicate 3-bp segments of nucleotide sequence identity between the junction and 3′ or 5′ donor sequences that flank a 20-bp inversion.

TABLE 2

Site/motif	Sequence[a]	Source
Vertebrate/plant topoisomerase I consensus cleavage sites	CAT	Been *et al.*, 1984
	CTY	Been *et al.*, 1984
	GTY	Been *et al.*, 1984
	RAT	Been *et al.*, 1984
Vaccinia topoisomerase I consensus cleavage site	YCCTT	Shuman, 1991
Vert./*Drosophila* topoisomerase II consensus cleavage sites	RNYNNCNNGYNGKTNYNY (v)	Spitzner and Muller, 1988
	GTNWAYATTNATNNR (D)	Sander and Hsieh, 1985
Polypurine runs	$(Y)_{n\geq 5}$	Konopka, 1988
Polypyrimidine runs	$(R)_{n\geq 5}$	Konopka, 1988
Alternating pyrimidine-purine runs	RYRYR	Konopka, 1988
Alternating purine-pyrimidine runs	YRYRY	Konopka, 1988
DNA pol-α pause sites	GAG	Weaver and Depamphilis, 1982
	GCS	Weaver and Depamphilis, 1982
	ACG	Weaver and Depamphilis, 1982
DNA pol-α frameshift hot spots	TCCCCC	Kunkel, 1985
	CTGGCG	Kunkel, 1985
DNA pol-β frameshift hot spots	ACCCWR	Kunkel, 1985
DNA pols-α/β frameshift hot spots	TGGNGT	Kunkel, 1985
	ACCCCA	Kunkel, 1985
Chi and chi-like sequences	GCTGGTGG	Dewyse and Bradley, 1991
	CCWCCWGC	Dewyse and Bradley, 1991
Human deletion hotspot	TGRRKM	Krawczak and Cooper, 1991
Murine MHC recombination hotspot	$(CAGR)_{n\geq 2}$	Steinmetz *et al.*, 1987
Consensus Ig switch region	TGGGG	Krawczak and Cooper, 1991
Ig/TCR recombinase heptamer	CACAGTG	Max, 1989
Ig/TCR recombinase nonamer	ACAAAAACC	Fuscoe *et al.*, 1991
Murine Parvovirus recomb. hotspot	CTWTTY	Hogan and Faust, 1986
Murine LTR recomb. hotspot	TGGAAATCCC	Edelmann *et al.*, 1989
Human fragile-X breakpoint cluster	$(CGG)_{n\geq 2}$	Verkerk *et al.*, 1991
Human hypervariable minisatellites		
Core sequence	GGAGGTGGGCAGGARG	Wahls *et al.*, 1990
Recombination hotspot	AGAGGTGGGCAGGTGG	Wahls *et al.*, 1990
Alu elements	*	Kariya *et al.*, 1987
LINE elements	*	Hutchinson *et al.*, 1989
Donehower repeats	*	Donehower *et al.*, 1989

[a] The designation for ambiguous or degenerate nucleotides follows the IUB convention: M, A or C; R, A or G; W, A or T; S, C or G; Y, C or T; and K, G or T. (*) Sequence not shown due to length. Direct and inverted or palindromic repeats and symmetric sequences (e.g., AACGCAA; see Krawczak and Cooper, 1991) were identified and analyzed visually, and thus are not included in Table 2.

tions ranging from 57 bp to 19.3 kb and three associated DNA inversions of 711, 368, and 20 bp. The junction region sequences from these mutants could be grouped into three classes on the basis of their sequence characteristics (Figs. 3–6). Each mutant contained a deletion of one or more *HPRT* exons and thus genetically inactivated the *HPRT* gene. No consistent difference in junction structure or complexity was observed between Werner syndrome- or HL60-derived deletions, suggesting that similar or identical pathways were used to generate deletions in both HL60 and Werner syndrome cells. Virtually all of these deletions and inversions appear to have arisen by the nonhomologous recombination of donor DNA sequences that share little nucleotide sequence identity and thus are similar to rearrangements observed in other human genes (Roth and Wilson, 1988; Konopka, 1988; Meuth, 1989, 1990).

The alignment and comparison of junction and donor sequences for each deletion revealed several sequence elements at or near many donor breakpoints of potential mechanistic importance (Figs. 3, 4, and 6). Short direct and inverted repeats were present at or flanking many of the junctions or donor breakpoints (see, e.g., PSV811-46R1, HL60-4F53, or the HL60-FB5 3′ junctions). In two of these junction sequences, the insertion of novel nucleotides created direct repeats between junction sequences and sequences immediately 3′ to the junction region (PSV811-46R1 and HL60-4F53; Fig. 4). This type of direct repeat is thought to arise in both procaryotes and eucaryotes by the slippage or transient misalignment of template and primer strands during DNA replication that allows the templated insertion of additional nucleotides (Roth *et al.*, 1985; Kunkel, 1990). A transient misalignment mechanism can explain the production of direct repeats 3′ to the PSV811-46R1 and HL60-4F53 junction sequences, although this mechanism does not explain how the 3.1- and 3.4-kb deletions associated with these insertions were generated. Two additional mutations found adjacent to junctions could also be explained by the transient misalignment of GTTT (HL60-

4F14a 5′) or GTT (PSV811-46R1) repeats (Figs. 4 and 6).

The complexity of the three tandem deletion-rearrangement mutants (HL60-4F14a and -FB5 and PSV811-46R2) was not appreciated until the complete sequence of the deletion junction regions from these mutants was obtained. The 5′ intra-*Alu* deletion in HL40-4F14a has an unusual 42-bp region of nucleotide sequence identity between donor strands, suggesting the occurrence of homologous pairing between donor sequences, while the 3′ 1501-bp deletion is between successive *Alu* repeats within *HPRT* that as aligned share little nucleotide sequence identity. The mutants PSV811-46R2 and HL60-FB5 were yet more complicated, having tandem deletions associated with 1 or 2 DNA inversions, respectively. Mammalian cells have particularly efficient mechanisms for joining unrelated DNA ends (see, e.g., Roth and Wilson, 1988), and thus it is tempting to speculate that all three of these mutations arose by the joining of a collection of closely spaced DNA ends generated at a disrupted DNA replication, recombination, or repair site. We anticipate that other mutations displaying this degree of complexity will be found when additional human somatic gene rearrangements are examined at the nucleotide sequence level.

We determined whether there was a statistically significant association between any of 33 sequence elements or motifs (Table 2) and donor breakpoints or junctions by comparing the distributions of breakpoint-to-element and element-to-element lengths in the *HPRT* locus. The sequence elements or motifs with which we performed this analysis had been previously implicated in chromosomal breakage, rearrangement, or deletion in one or more eucaryotic gene (Table 2; reviewed in Steinmetz *et al.*, 1987; Murnane, 1990; Krawczak and Cooper, 1991). Of these elements only the vertebrate topoisomerase I consensus cleavage sequence CTY (Y = C or T; Been *et al.*, 1984) showed greater-than-chance association with deletion donor breakpoints.

Topoisomerase I is believed to promote nonhomologous recombination by virtue of DNA strand breakage in DNA regions that are not fully base-paired (Champoux and Bullock, 1988). Enzymatically active topoisomerase I can remain covalently attached to the 3′ nucleotide of the cleavage site and can join this free DNA end to other DNA molecules (see Champoux and Bullock, 1988). The presence of these sites at or near many mammalian nonhomologous recombination breakpoints (Champoux and Bullock, 1988; Konopka, 1988) suggests that chromosomal breakage may be a rate-limiting step in the initiation of nonhomologous recombination *in vivo*. This idea could be tested by determining whether topoisomerase I overexpression promotes mutagenesis or DNA rearrangements in mammalian cells.

One surprise in our analyses was the absence of deletions generated by recombination between *Alu* repeats in the *HPRT* locus. We had anticipated, from molecular analyses of disease-associated germinal deletions in different human genes (see, e.g., Lehrman *et al.*, 1987; Myerowitz and Hogikyan, 1987; Markert *et al.*, 1988; Miura *et al.*, 1989; Huang *et al.*, 1989; Berkvens *et al.*, 1990; reviewed in Meuth, 1989), that this pathway might be used to generate deletions in the *Alu*-rich *HPRT* locus. One plausible explanation for the absence of *Alu–Alu* deletions is that *Alu* repeats contained in the human *HPRT* locus have only short (≤30 bp) segments of nucleotide sequence identity rather than the 200–300 bp of sequence identity needed to promote efficient intrachromosomal recombination in mammalian cells (reviewed in Bollag *et al.*, 1989). Another explanation for the paucity of *Alu–Alu* deletions in the *HPRT* gene of human somatic cells is that the *Alu–Alu* deletion pathway is used preferentially in germ cells, as opposed to somatic cells. We are now testing this intriguing possibility by determining the junction sequences of several human germinal *HPRT* deletions from Lesch–Nyhan patients (Yang *et al.*, 1984).

A better understanding of the pathways that control deletion generation in somatic and germ cells will be of intrinsic interest and may have practical importance: deletions play a role in the genesis of heritable human disease and play both physiologic and pathologic roles in human somatic cells *in vivo*. The human *HPRT* locus offers several distinct advantages for analyzing the mechanisms that generate human germinal and somatic deletions: both germinal and somatic mutations can be recovered and compared; and the somatic cell, molecular, and reverse genetics of the human *HPRT* locus are sufficiently well-developed to allow mechanistic hypotheses to be formulated and tested.

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