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Molecular Structure and Genetic Stability of Human Hypoxanthine Phosphoribosyltransferase (*HPRT*) Gene Duplications

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We have determined the genetic stability of three independent intragenic human *HPRT* gene duplications and the structure of each duplication at the nucleotide sequence level. Two of the duplications were isolated as spontaneous mutations from the HL60 human myeloid leukemia cell line, while the third was originally identified in a Lesch–Nyhan patient. All three duplications are genetically unstable and have a reversion rate approximately 100-fold higher than the rate of duplication formation. The molecular structures of these duplications are similar, with direct duplication of *HPRT* exons 2 and 3 and of 6.8 kb (HL60 duplications) or 13.7 kb (Lesch–Nyhan duplication) of surrounding *HPRT* sequence. Nucleotide sequence analyses of duplication junctions revealed that the HL60-derived duplications were generated by unequal homologous recombination between clusters of *Alu* repeats contained in *HPRT* introns 1 and 3, while the Lesch–Nyhan duplication was generated by the nonhomologous insertion of duplicated *HPRT* DNA into *HPRT* intron 1. These results suggest that duplication substrates of different lengths can be generated from the human *HPRT* exon 2–3 region and can undergo either homologous or nonhomologous recombination with the *HPRT* locus to form gene duplications.

INTRODUCTION

Gene duplication has played an important role in the evolution of individual genes and in the generation and diversification of multigene families (reviewed in Li, 1983; Nei, 1987). Gene duplications have also been identified as pathogenetic mutations in different human genetic diseases (reviewed in Cooper and Schmidtke, 1991). Disease-associated duplications have been identified, for example, in patients with Lesch–Nyhan syndrome (Yang *et al.*, 1984, 1988), hemophilia A and B (Gitschier, 1988; Murru *et al.*, 1990), familial hypercholesterolemia (Lehrman *et al.*, 1987; Lelli *et al.*, 1991), lipoprotein lipase deficiency (Devlin *et al.*, 1990), and Duchenne and Becker muscular dystrophy (Hu *et al.*, 1988, 1991). Molecular analyses of these disease-associated duplications have revealed many partial gene duplications that result in genetic inactivation of the affected allele.

Gene duplication occurs in somatic cells as well as in germ cells and may play an important role in initiating gene amplification by generating the inverted or direct repeat units contained in many amplified chromosomal regions (Meuth, 1989, 1990; Stark *et al.*, 1989). The duplicated genes contained in amplification units may play pathogenetically important roles in tumorigenesis or tumor progression when overexpressed: examples include members of the *myc* and *mdr* gene families (Endicott and Ling, 1989; Schwab and Amler, 1990).

We do not have a detailed understanding of the mutation pathways and nucleotide sequence substrates that participate in gene duplication or amplification in mammalian cells. Duplications rarely represent more than a few percent of mutations in mammalian locus-specific germinal and somatic mutation collections (Nicklas *et al.*, 1987; Fukuchi *et al.*, 1989; Monnat, 1989; Meuth, 1990; Cooper and Schmidtke, 1991; Hu *et al.*, 1991), and thus it has been difficult to collect multiple independent duplications at a single genetic locus for molecular analysis. The inherent genetic redundancy of duplications and their often large size (Meuth, 1989, 1990; Stark *et al.*, 1989) have also hampered attempts to determine the molecular structure of duplication and amplification events at the nucleotide sequence level.

To begin to elucidate mutation pathways that give rise to human gene duplications, we have determined the structure of three intragenic human *HPRT* duplications at the nucleotide sequence level. One of these duplications was first identified as a germinal mutation in a Lesch–Nyhan patient (Yang *et al.*, 1984). The remaining two duplications were isolated as independent, spontaneous somatic mutations from the HL60 human myeloid leukemia cell line (Monnat, 1989). Tumor cells and cell lines such as HL60 (see, for example, Collins and Groudine, 1982) undergo gene amplification much more frequently than normal diploid cells (Stark *et al.*, 1989; Tlsty, 1990; Wright *et al.*, 1990) and thus might be able

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to generate amplification intermediates such as duplications more readily than normal cells. We have also used the well-developed somatic cell genetics of the *HPRT* locus to determine the genetic stability of these three duplications.

MATERIALS AND METHODS

Cell culture. The independent 6-thioguanine (TG)-resistant duplication mutants 3F22 and HG4 were isolated from the HL60 human myeloid leukemia cell line (Collins *et al.*, 1977) and characterized by blot hybridization analysis as previously described (Monnat, 1989). The Epstein–Barr virus-transformed lymphoblastoid cell line GM6804, derived from a Lesch–Nyhan patient with a variant clinical phenotype (Gottlieb *et al.*, 1982), was obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). All three cell lines and control HL60 cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and in the case of duplication mutants 30 μ*M* TG, in a humidified 37°C, 7% CO_2/93% air incubator. Cell lines were tested on multiple occasions and found to be negative for the presence of *Mycoplasma* infection using a 4′,6-diamidino-2-phenylindole (DAPI) fluorescence microscopy assay (after Chen, 1977).

The colony-forming efficiencies (CFE) of duplication and control cell lines were determined by dilution cloning (3F22, HG4, and GM6804) or in reconstruction experiments (3F22, HG4) as previously described (Monnat, 1989). All of these determinations were performed with 96-well microtiter plates in which the center 60 wells were used for cell plating and perimeter wells were filled with sterile water to retard the evaporation of medium. Dilution cloning experiments were performed by plating an average of 2 or 5 cells (3F22 and HG4) or 10 to 100 cells (GM6804) per well in each of 60 or 120 round-bottom microtiter plate wells in 100–200 μl of complete RPMI medium (RPMI1640 medium supplemented with 15% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin). Reconstruction experiments were performed using HAT (100 μ*M* hypoxanthine, 1 μ*M* aminopterin, and 20 μ*M* thymidine)-resistant sublines that were isolated as spontaneous HAT-resistant revertants from 3F22 and HG4 (3F22-A3D and HG4-3A2; unpublished results). In each reconstruction experiment an average of 4 or 10 HAT-resistant cells were plated in the presence of 5×10^4 3F22 or HG4 cells in each of 60 flat-bottom microtiter plate wells in 100 μl of complete RPMI medium supplemented with HAT. Cells were fed once by adding 90–100 μl/well of HAT medium 7–10 days after plating, and wells containing colonies of $>10^3$ viable cells after 3 weeks growth were identified by microscopy. CFEs were calculated using the formula $CFE = -\ln(P_0)/n$, where P_0 is the fraction of wells containing no colonies and n is the mean number of cells plated per well.

The frequency of HAT-resistant revertant cells in 3F22 and HG4 was quantified by determining the CFE of cells plated in HAT medium. Cell cultures used for these determinations had been grown in the absence of TG for a minimum of 20 population doublings to allow the accumulation of HAT-resistant revertants. Reversion frequency determinations were performed by plating an average of 5×10^4 cells/well in each of 600 flat-bottom microtiter plate wells in 200 μl of HAT medium. Cells were fed once by adding 100 μl/well of HAT medium after 7–10 days growth. Wells containing colonies of $>10^3$ viable cells after 3 weeks of growth in the presence of HAT were identified by microscopy. Several HAT-resistant revertants from each duplication cell line were isolated and grown for blot hybridization analysis of *HPRT* gene structure (see below).

The reversion rate of *HPRT* duplication cell lines 3F22, HG4, and GM6804 to HAT resistance was determined by a modification of the fluctuation test of Luria and Delbrück (1943) (Monnat, 1989). Reversion rate determinations were performed with 10 replicate cultures/mutant that were each grown from an inoculum of 100 cells to approximately 10^7 cells in the absence of TG. Each replicate culture was then pelleted and resuspended in HAT medium before plating an average of 5×10^4 cells in 200 μl of HAT medium in each of 120 flat-bottom microtiter plate wells. Cells were fed once by adding 100 μl of HAT medium 7 days after plating, and wells containing colonies of $>10^3$ viable cells after 21 days growth in the presence of HAT were identified by microscopy. Reversion rates were calculated using the formula $a = CaN_t/CN_t$, where a is the mutation rate per cell per generation, C is the number of replicate cultures, and N_t is the number of cells plated per replicate culture. CaN_t values were estimated from the product of Cr, where r is the average number of mutant cells per culture (Capizzi and Jameson, 1973). All rates were then corrected for cell line CFE determined as described above.

TABLE 1

Oligonucleotide Primers Used for Human *HPRT* Duplication Analysis

Template[a]	(−) primer/ *n*-mer[b]	(+) primer/ *n*-mer	Seq primer/ *n*-mer
3F22/HG4	19497(−)/21	14953(+)/21	20657(−)/21
GM6804-5′	7046(−)/24	11852(+)/23	
GM6804-3′	24981(−)/17	9530(+)/19	
cDNAs	99(−)/22	757(+)/28	

[a] Templates were high-molecular-weight DNA (3F22, HG4, and GM6804) or cDNA prepared from each duplication mutant by the reverse transcription of total cellular poly(A)$^+$ RNA. GM6804 5′ and 3′ templates refer to the 5′ intron 1–intron 1 and 3′ intron 3–intron 1 duplication novel junctions in GM6804, respectively.

[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; genomic DNA primers), or in the human *HPRT* cDNA sequence (Jolly *et al.*, 1983; cDNA primers). (+) 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. The cDNA primers contain 1 (99(−)) or 2(757(+)) nucleotide substitutions when compared with the human *HPRT* cDNA sequence (Jolly *et al.*, 1983). "seq" primers, DNA sequencing primers.

Blot hybridization analyses. Northern and Southern blot hybridization analyses were performed as previously described (Monnat, 1989). Hybridization probes consisted of the coding region or selected exons derived from a cloned human *HPRT* cDNA (Fukuchi *et al.*, 1989; Jolly *et al.*, 1983) and cloned human *HPRT* locus intron segments (Jolly *et al.*, 1982; Patel *et al.*, 1986). Probes were radiolabeled with [^{32}P]dCTP (3000 Ci/mmol, Amersham) by random oligonucleotide-primed DNA synthesis (Feinberg and Vogelstein, 1983).

Partial digest–end label mapping of *HPRT* gene structure in duplication mutants and controls was performed by a modification of the method of Smith and Birnstiel (1976). DNA (100 μg) was digested with the restriction endonuclease *Bam*HI (1 unit/μg DNA) at 37°C for 16 h in 220 μl of buffer recommended by the manufacturer. Aliquots of *Bam*HI-digested DNA were then digested to differing degrees of completion with the restriction endonuclease *Pst*I (1–0.016 unit/μg). After 4 h of digestion at 30°C, samples were heated to 80°C for 5 min prior to gel electrophoresis and capillary blotting onto a nylon hybridization membrane, as previously described (Monnat, 1989). Hybridizations were performed with a 1.7-kb probe derived from the 5′ end of the human *HPRT* intron 1 (pPB1.7; Jolly *et al.*, 1982). Radiolabeled probe (50 ng) and 200 μg of alkali-sheared human lymphoblastoid cell line DNA were boiled for 10 min in 200 μl of 5× SSC, then incubated at 68°C for 20 min to remove repeated sequences from the probe preparation prior to blot hybridization analyses (Sealey *et al.*, 1985; Vogelstein *et al.*, 1987). Blot hybridization and wash conditions for partial digest–end label experiments were as previously described (Monnat, 1989) with the exception of the final wash step, which was at 65°C for 1 h in 0.1× SSC/0.5% SDS.

PCR amplification of HPRT cDNAs and of HPRT duplication novel junctions. Oligonucleotide primers for cDNA syntheses, amplification, and sequencing (Table 1) were synthesized by Operon Technolo-

gies (Alameda, CA) or by the Howard Hughes and 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 (TE) buffer.

First-strand cDNA syntheses were performed using an Amersham cDNA synthesis kit (Amersham RNP1256). Reactions contained 2.5 μg poly(A)$^+$ RNA primed with 2.5 μg oligo(dT) (HG4) or with 200 ng of human *HPRT*-specific oligonucleotide primer (757(+), HL60 and 3F22; see Table 1). Two human *HPRT* cDNA primers, 99(−) and 757(+), were used to amplify *HPRT* cDNA coding regions from reverse transcription reactions. Amplification reactions contained 25% of each cDNA synthesis reaction, 200 ng of each primer, and 1 unit of Amplitaq (Perkin–Elmer/Cetus) in 100 μl of 10 m*M* Tris (pH 8.3), 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 100 μg/ml gelatin, and 200 μ*M* dNTPs. Amplification reactions (35 cycles) were performed in 0.65 ml microfuge tubes with a paraffin oil overlay in a Perkin–Elmer/Cetus Thermal Cycler. Each cycle consisted of a 94°C, 1-min denaturation step followed by 69°C, 1-min primer annealing, and 72°C, 1-min extension steps. Reaction products were extracted once with an equal volume of phenol/chloroform (24/1), ethanol-precipitated, and digested with the restriction endonucleases *Bam*HI and *Eco*RI prior to size fractionation on a 5% polyacrylamide, 1× TBE gel. Fragments corresponding to the predicted cDNA product sizes of approximately 1000 bp (duplications) or 700 bp (wildtype) were isolated by electroelution, then ligated into pBluescriptII KS + plasmid (Stratagene) for transformation into the *Escherichia coli* host strain TB1. Deletion mutants were constructed from cloned cDNAs by digesting cDNA plasmids (5 μg each) with the restriction endonuclease *Hin*dIII, which cleaves at the human *HPRT* cDNA exon 6–7 junction and in a 3′ vector polylinker sequence. *Hin*dIII-cleaved plasmids were isolated from low-melting-temperature agarose gels, recyclized, and ligated prior to transformation into the *E. coli* host strain TB1.

Duplication junctions were amplified for DNA sequence analyses with human *HPRT*-specific oligonucleotide primers (Table 1). Amplification reactions (35 cycles) were performed as described above using 500 ng of cellular DNA template. Each amplification cycle consisted of a 94°C, 45-s denaturation step, followed by 42–58°C, 45-s primer annealing, and 72°C, 45-s to 3.5-min extension steps. DNA from HL60 cells was used as a control template. Amplification products were analyzed by electrophoresis on 1% (w/v) agarose gels prepared in 1× TBE buffer, and their identity was verified by restriction endonuclease mapping or blot hybridization with human *HPRT*-specific probes. Junction fragments were cloned into pBluescriptII to facilitate DNA sequence analyses.

DNA sequence analysis of complementary and genomic DNAs. Duplication and control *HPRT* cDNAs and *HPRT* duplication junctions were sequenced by the dideoxy chain termination method using modified T7 DNA polymerase (Sequenase, USB). Sequencing primers (Table 1 and pBluescriptII universal sequencing primers (Stratagene)) 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 fragment that had been gel-purified using GeneClean (BIO101) or to 3 μg of alkali-denatured plasmid DNA by heating primer-template mixes to 95–100°C for 5 min, then immediately placing reactions in a dry ice/ethanol bath. Sequencing reactions were performed by adding primed templates directly to ddNTP termination mixes. Completed reactions were run on 8% polyacrylamide/7 *M* urea gels and dried prior to autoradiography as previously described (Monnat and Loeb, 1985). 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).

RESULTS

Colony-forming efficiency and genetic stability of duplications. The CFEs of duplication mutants determined by dilution cloning were 36% (3F22), 34% (HG4), and 2.2% (GM6804). CFEs of HL60 HAT-resistant sublines 3F22-A3D and HG4-3A2, determined in reconstruction experiments using parental 3F22 and HG4 cells, were 42% (3F22-A3D) and 48% (HG4-3A2). The frequencies of HAT-resistant cells in 3F22 and HG4 grown for greater than 20 population doublings in the absence of TG were 9.6×10^{-7} (3F22) and 1.8×10^{-6} (HG4). The reversion rates per cell per generation of 3F22, HG4, and GM6804 to HAT resistance, determined by a modification of the mean method of Luria and Delbrück (1943) (Monnat, 1989) and corrected for CFE, were 6.6×10^{-6} (3F22), 4.0×10^{-6} (HG4), and 9.6×10^{-5} (GM6804).

Blot hybridization analysis of duplication mutant HPRT mRNAs and sequence analysis of derived cDNAs. Northern blot analyses of *HPRT* mRNA in 3F22 and HG4 revealed single RNA species approximately 1.9 kb long, or 300 bases longer than the 1.6-kb *HPRT* mRNA observed in control cells (Monnat, 1989; additional data not shown). The additional sequence information contained in 3F22 and HG4 *HPRT* mRNAs was characterized by sequencing *HPRT* cDNA clones amplified from each mutant and from control cells. Two cDNA amplification products were observed: a 1-kb fragment from 3F22 and HG4 cells and a 700-bp fragment from HL60 control cells (data not shown). The coding region sequences of *HPRT* cDNAs from 3F22 and HG4 contained identical direct, exact duplications of exons 2 and 3. The coding region sequence of an *HPRT* cDNA amplified from HL60 control cells was identical to the human *HPRT* cDNA sequence reported by Jolly *et al.* (1983). The duplicated exons found in 3F22 and HG4 cDNAs encode *HPRT* amino acid residues 10–106. Their presence genetically inactivates *HPRT* in both 3F22 and HG4 (<1% of control activity (Monnat, 1989)).

Southern blot analyses were used to determine *HPRT* gene structure in duplication mutants. DNAs were digested with either one or two restriction endonucleases prior to blot hybridization analysis with human *HPRT* probes containing the complete cDNA coding region, selected exons, or segments of *HPRT* introns 1 or 3 (Fukuchi *et al.*, 1989; Jolly *et al.*, 1983; Patel *et al.*, 1986; Yang *et al.*, 1988). Blot hybridization analyses of *HPRT* gene structure in 3F22 and HG4 with a *HPRT* cDNA probe containing exons 1–9 revealed novel-sized restriction fragments in DNA from both mutants when digested with restriction endonucleases *Eco*RI (7 kb), *Pst*I (2.65 kb), or *Xba*I (6.9 kb), although not with *Bgl*II, *Hin*dIII, or *Sac*I. We also observed a consistent increase in hybridization intensity of exon 3-containing *Bgl*II (3.4 kb) and *Pst*I (1.3 kb) restriction fragments in both mutants (Fig. 1A). When a human *HPRT* exon 3 hybridization probe was used, we observed novel-sized *Pvu*II (6.8 kb), *Pvu*II + *Eco*RV (6.8 kb), and *Pvu*II + *Xba*I (6.2 kb) fragments in 3F22 and HG4 DNAs, although not in 3F22 or HG4 DNA digested with *Pvu*II + *Bam*HI, +*Bgl*II, +*Eco*RI, +*Hin*dIII, or +*Pst*I (additional data not shown).

Southern blot analysis of *HPRT* gene structure in GM8604 with an *HPRT* cDNA probe containing exons

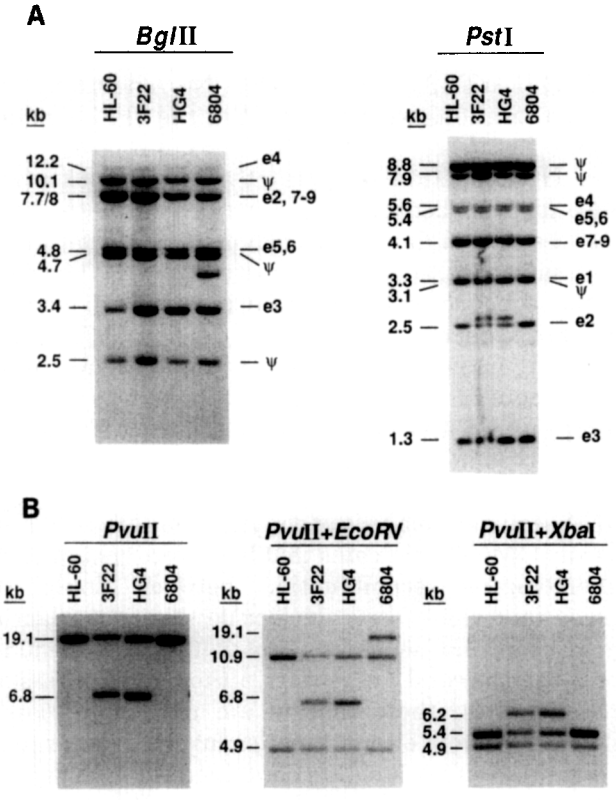

FIG. 1. Southern blot hybridization analyses of human *HPRT* gene duplications. DNA from control HL60 cells or from duplication mutants 3F22, HG4, or GM6804 was digested with one or two restriction endonucleases prior to blot hybridization analysis with probes containing the complete human *HPRT* cDNA coding region (**A**) or *HPRT* exon 3 only (**B**). (**A**) A novel-sized 4-kb *Bgl*II restriction fragment was observed in GM6804 DNA. Novel-sized (2.7 kb) *Pst*I restriction fragments were observed in 3F22 and HG4 DNA. An increase in hybridization intensity was observed for normal-sized exon 3-containing *Pst*I (1.3 kb) and *Bgl*II (3.4 kb) restriction fragments from all three duplications. 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. (**B**) Novel-sized restriction fragments were observed in 3F22 and HG4 DNAs digested with *Pvu*II (6.8 kb), *Pvu*II + *Eco*RV (6.8 kb) and *Pvu*II + *Xba*I (6.2 kb). A novel-sized *Pvu*II + *Eco*RV fragment (approx 19 kb) was observed in GM6804. Increased hybridization intensities of normal-sized GM6804 *Pvu*II (19.1 kb) and *Pvu*II + *Xba*I (5.4 kb) fragments were also observed. The 4.8- to 4.9-kb fragment observed in *Pvu*II + *Eco*RV and *Pvu*II + *Xba*I panels are derived from autosomal human *HPRT* pseudogene(s). Restriction fragment sizes are given in kilobases at the left. The hybridization conditions used do not efficiently display *HPRT* exon 1-containing restriction fragments, which appear as weak (e.g., *Pst*I) or absent (e.g., *Bgl*II) bands in these blots.

1–9 revealed a novel-sized restriction fragment with DNA digested with *Bgl*II (4.2 kb; Fig. 1A), although not with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sac*I, or *Xba*I (*Pst*I results shown in Fig. 1A; other data not shown). We also observed an increase in hybridization intensity of normal-sized restriction fragments containing *HPRT* exons 2 and 3 (*Eco*RI, 8.4 kb; *Pst*I, 2.5 and 1.3 kb; and *Xba*I, 5.4 kb). (*Pst*I results shown in Fig. 1A; other data not shown.) When a human *HPRT* exon 3 hybridization probe was used we observed a novel-sized restriction fragment in GM6804 DNA digested with *Pvu*II + *Eco*RV (approximately 19 kb). No additional novel-sized restriction fragments were observed in GM6804 DNA digested with *Pvu*II + *Bam*HI, +*Bgl*II, +*Eco*RI, +*Hin*dIII, +*Pst*I, or +*Xba*I (*Eco*RV and *Xba*I data shown in Fig. 1B; other data not shown). This probe also revealed increased hybridization intensity of the normal-sized 5.4-kb *Xba*I fragment that contains *HPRT* exon 3 in *Pvu*II + *Xba*I-digested DNA (Fig. 1.B.).

We next used a modification of the partial digest–end label mapping method of Smith and Birnstiel (1976) to further delineate *HPRT* gene structure in the duplication mutants. DNA from each mutant and from HL60 cells was digested to completion with *Bam*HI, which produces a 20-kb restriction fragment extending from the 5′ end of intron 1 to intron 3. Previous blot hybridization analyses had revealed that none of the duplications contained a known intron 1 *Bam*HI polymorphism that would interfere with this approach (Nussbaum *et al.*, 1983; data not shown). *Bam*HI-digested DNA was then digested to different degrees of completion with the restriction endonuclease *Pst*I prior to blot hybridization analysis with a probe derived from the 5′ end of intron 1 (pPB1.7; Jolly *et al.*, 1982). All predicted *Pst*I partial digest restriction fragments were observed in HL60 control DNA at *Pst*I concentrations of <0.032 unit/μg DNA. *Pst*I partial digest fragments observed in 3F22 and HG4 were virtually identical in size to those observed in control DNA (Fig. 2; 3F22 data only shown). The *Pst*I partial digest pattern observed in GM6804, in contrast, differed from that observed in control and in 3F22 or HG4 cells in fragment size and spacing after the second 3.3-kb *Pst*I partial digest fragment (Fig. 2).

A combination of conventional and end label blot hybridization and cDNA sequencing results allowed us to construct unambiguous and internally consistent models of *HPRT* gene structure in each duplication mutant (Fig. 3). 3F22 and HG4 appeared to contain very similar direct duplications of a 6.8-kb segment of *HPRT* exon 2–3 region DNA with single intron 3–intron 1 novel junctions. GM6804, in contrast, appeared to contain a direct duplication of 13.7 kb of *HPRT* exon 2–3 region DNA that had been inserted into *HPRT* intron 1 with the creation of 5′ intron 1–intron 1 and 3′ intron 3–intron 1 novel junctions (Fig. 3).

Blot hybridization analysis of HAT-resistant revertants from 3F22 and HG4. Southern blot hybridization analyses of 3F22 and HG4 HAT-resistant revertants with human *HPRT* cDNA and intron 3 probes revealed the loss of duplication-associated *Pst*I and *Bam*HI restriction fragments (data not shown). We could not determine whether the *HPRT* gene structure of revertants had been completely restored or whether our HAT-revertant sublines represented independent mutational events.

Amplification and DNA sequence analysis of duplication-associated novel junctions. Single junction fragments of 2.2 kb were amplified from 3F22 and HG4 using the same primer pair, while junction fragments of 1.3 kb (5′ junction) and 1.8 kb (3′ junction) were amplified from

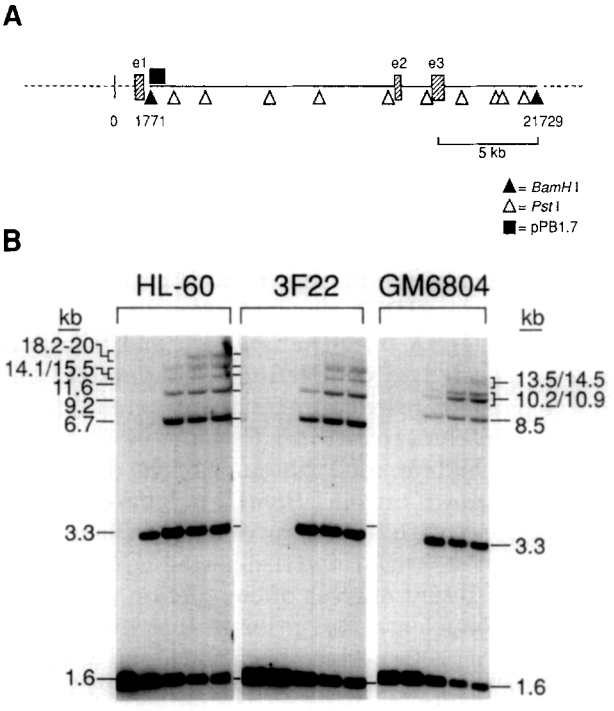

FIG. 2. Partial digest–end label mapping of *HPRT* gene structure in three human *HPRT* gene duplications. (**A**) *Bam*HI (▲) digestion of human DNA produces a 20-kb restriction fragment from the 5′ half of the *HPRT* gene (horizontal solid line, top). *Pst*I (△) cleaves at 10 positions within this fragment. *Pst*I partial digest products that contain the original 5′ *Bam*HI end and a 3′ *Pst*I end can be selectively detected by blot hybridization with probe pPB1.7 (■) derived from the 5′ end of intron 1 (Jolly *et al.*, 1982). Nucleotide sequence numbering is after Edwards *et al.* (1990). (**B**) Partial digest–end label blot hybridization analyses of DNAs from control (HL60) cells and from duplication mutants 3F22 and HG4 (3F22 only is shown) were identical to beyond the location of *HPRT* exon 3 (*HPRT* nucleotide 16786). GM6804 DNA, in contrast, shows alterations in *Pst*I partial digest fragment size and spacing after the second 3.3-kb *Pst*I partial fragment. The *Pst*I concentration range shown is 0.25 unit/μg (**left**) to 0.016 unit/μg (**right**) DNA in 4-h digests at 30°C.

GM6804 using two different primer pairs (Table 1). DNA sequence analyses of the 3F22 and HG4 junction fragments indicate that each was generated by unequal homologous recombination between *Alu* repeats in *HPRT* introns 1 and 3. These junctions were separated by only 110 bp in *HPRT* donor sequences containing four pairs of *Alu* repeat elements with the potential for direct or inverted base pairing (Fig. 4B). The substantial degree of nucleotide sequence identity between these donor regions and their potential for base pairing is readily apparent in dot-matrix homology comparisons (Fig. 4C).

The 6-bp junction region in 3F22 contains A → G and T → A substitutions and is flanked by 19-bp (5′) and 22-bp (3′) blocks of sequence identity between junction and donor duplexes (Fig. 4A). A third substitution, a G → C transversion, was also observed 98 bp upstream, 8 bp 3′ to the HG4 junction region (Fig. 4B). These base substitutions are not sequence polymorphisms in HL60 cells and thus are likely to have been generated during duplication formation. Several short direct and inverted repeat sequences are present in the 3F22 junction region, as is an AT-rich direct repeat contained in the 22-bp block of sequence identity 3′ to the junction ($AA(T)_5GTA(T)_5AGT$)(Fig. 4A). No additional sequence alterations were observed in the 3F22 junction region.

The HG4 junction consists of a 24-bp block of sequence identity between the junction and both donor sequences that is flanked by regions of substantial, although imperfect, sequence identity (Fig. 4A). The most notable sequence motif observed in the HG4 junction and donor sequences is an AT-rich region ($A(T)_{12}$ in 5′ donor and junction sequences and $(T)_5G(T)_6(A)_3$ in the 3′ donor sequence) that is located 13–15 bp 5′ to the junction region (Fig. 4A). No additional sequence alterations were observed in the HG4 junction sequence or in flanking DNA.

DNA sequence analysis of the junction regions amplified from GM6804 indicates that both were generated by nonhomologous recombination between donor sequences that share little nucleotide sequence identity (Fig. 5). The donor sequences contributing to these junctions contain several *Alu* repeats, although in contrast to 3F22 and HG4, these repeats are not appropriately aligned to promote either direct or inverted donor pairing (Fig. 5). Neither junction contained AT-rich repeats similar to those we observed near the 3F22 and HG4 junctions. A 10- to 14-bp deletion of intron 1 DNA (nucleotides 7979–7988) was also observed when the two junction donor sequence sets were compared with junction sequences.

The nucleotide sequences of the 3F22, HG4, and GM6804 duplication junctions have been deposited with the EMBL/GenBank/DDBJ Data Libraries under Accession Nos. M84541–M84544. These duplication junction sequences are referenced to the human *HPRT* locus sequence determined by Edwards *et al.* (1990), Accession No. M26434.

DISCUSSION

We have used a combination of somatic cell and molecular genetic techniques to determine the genetic stability of three independent human *HPRT* gene duplications and their structures at the nucleotide sequence level. We isolated two of these duplications as spontaneous somatic mutations in replicate cultures derived from the HL60 human myeloid leukemia cell line (Collins *et al.*, 1977; Monnat, 1989). The third duplication was originally identified as a disease-associated germinal mutation in a Lesch–Nyhan patient (Gottlieb *et al.*, 1982; Yang *et al.*, 1984).

We determined the genetic stability of each duplication by quantifying the frequency of revertant cells and the reversion rate to HAT resistance. These forward and reverse mutation rates (Monnat, 1989; Penman *et al.*, 1983 and unpublished results), in conjunction with knowledge of the proportion of forward mutations at the *HPRT* locus that are duplications (4% in HL60 cells; Monnat, 1989), indicate that *HPRT* gene duplications

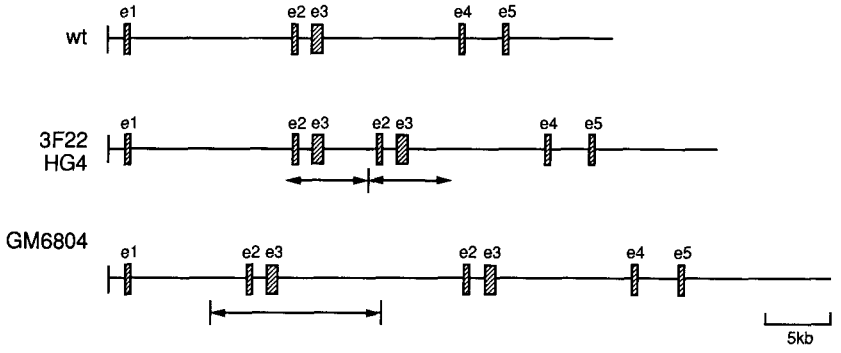

FIG. 3. Structural models of three human *HPRT* gene duplications. The structure of the 5′ half of the human *HPRT* gene (horizontal solid lines) in control cells (wt) and in *HPRT* duplication mutants was deduced from a combination of blot hybridization and cDNA sequence analyses. The 5′ (**left**) end of each gene is defined by the *Eco*RI restriction endonuclease cleavage site at *HPRT* nucleotide 1 (Edwards *et al.*, 1990), and the positions of exons in each gene are indicated by hatched boxes. The extent of the duplicated regions is indicated by the double arrow (↔), and the positions of duplication junctions are indicated by a vertical (|).

are genetically unstable and revert approximately 100-fold more rapidly than they are formed.

The genetic instability of *HPRT* gene duplications may have implications for the biology of Lesch–Nyhan syndrome. Molecular and phenotypic reversion of a disease-associated germinal *HPRT* duplication *in vivo* would generate cellular mosaicism in patient tissue. Reversion events early in embryogenesis might contribute $HPRT^+$ cells to many tissues and thus mitigate the Lesch–Nyhan phenotype. This mechanism has been suggested as an explanation for the comparatively mild clinical phenotype of hyperuricemia and choreoathetosis without mental retardation or self-mutilation of the Lesch–Nyhan patient from whom the GM6804 lymphoblastoid cell line was derived (Gottlieb *et al.*, 1982; Yang *et al.*, 1988). A corollary of this idea is that duplications or other easily revertable *HPRT* mutations may be overrepresented among Lesch–Nyhan patients having variant phenotypes. These predictions could be tested by determining the proportion of HAT-resistant peripheral blood lymphocytes or by determining the proportion of duplication and revertant *HPRT* genes in Lesch–Nyhan variant patients.

One method we used to help establish *HPRT* gene structure and locate duplication junctions was a modification of the partial digest–end label mapping procedure of Smith and Birnstiel (Smith and Birnstiel 1976; Fig. 2). These experiments demonstrated the feasibility of generating high-resolution restriction maps of 15- to 20-kb segments of single-copy human genes using unfractionated cellular DNA as starting material and suggest this mapping strategy may be useful for rapid molecular characterization of other human X- or Y-linked structural rearrangements.

We determined the nucleotide sequence of the junction(s) in each duplication to see if these junctions might share common—and perhaps mechanistically interesting—nucleotide sequence features (Figs. 3–5). Sequence analyses indicated that the 3F22 and HG4 junctions were generated by unequal homologous recombination between the same pair of *Alu* repeats flanking exons 2 and 3 (Fig. 4.). The duplication junctions in GM6804, in contrast, appear to have been generated by nonhomologous recombination between *HPRT* regions that demonstrate little sequence identity in association with a deletion of 10–14 bp (*HPRT* nucleotides 7979–7988) of intron 1 DNA (Fig. 5).

The molecular structures and nucleotide sequence alterations we identified in *HPRT* duplications 3F22, HG4, and GM6804 share structural and sequence similarities with other human gene duplications. Many well-characterized human germinal gene duplications are partial, direct duplications that appear to have been generated by unequal homologous recombination between *Alu* elements (see, e.g., Lehrman *et al.*, 1987) or by nonhomologous recombination between unique or repetitive DNA sequences within the duplicated gene (see, e.g., Maeda *et al.*, 1984; Devlin *et al.*, 1990; Murru *et al.*, 1990). In some genes, e.g., the Duchenne muscular dystrophy (DMD) locus, both mutation pathways appear to be used to generate duplications (Hu *et al.*, 1991). In contrast, duplications associated with gene amplification events are most often inverted duplications joined by an unduplicated central region that contains one or more nonhomologous recombination junctions (reviewed in Meuth, 1989, 1990; Stark *et al.*, 1989). An interesting exception to these observations has been seen in Vaccinia virus: most Vaccinia virus isolates resistant to hydroxyurea, an agent that inhibits DNA synthesis and promotes gene amplification, contain amplified *direct* repeat arrays of the ribonucleotide reductase small subunit (M2) gene (Slabaugh *et al.*, 1989). The initial direct M2 duplication appears to have been generated by nonhomologous recombination then amplified in independent isolates by repeated unequal crossing over. The mammalian *HPRT* locus is permissive of amplification, although it is not clear from reported examples whether *HPRT* amplification preferentially involves direct or inverted repeat amplification units (Fenwick *et al.*, 1984; Fuscoe *et al.*, 1983).

The duplications we have examined could have arisen by several mechanisms. For example, unequal crossing over between newly replicated sister chromatids could explain the origin of the HL60-derived duplications. A

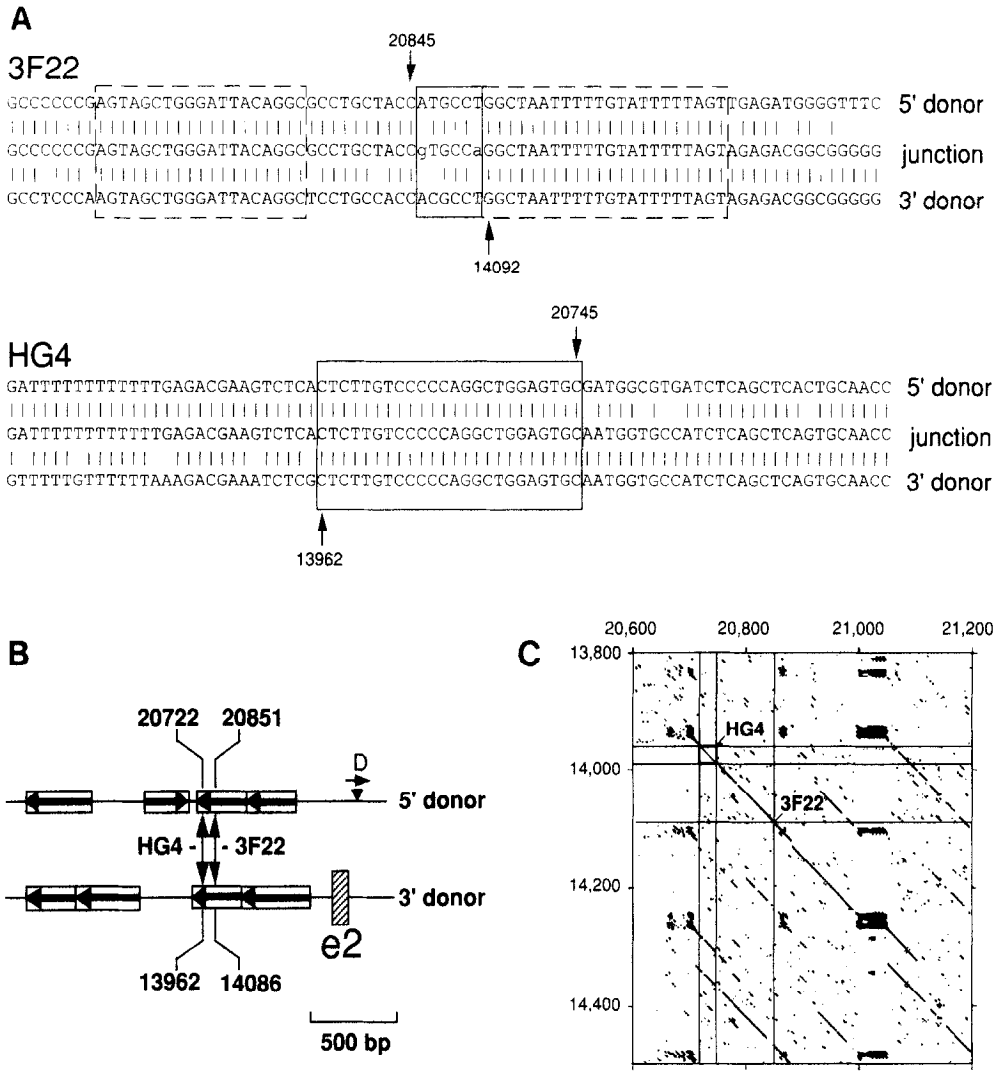

FIG. 4. Nucleotide sequence and structure of 3F22 and HG4 *HPRT* duplication junctions. (**A**) Junction sequences for 3F22 and HG4 are shown aligned with *HPRT* donor sequences. Crossover regions (solid-line boxes) are indicated for each junction, as are 19-bp 5′ and 22-bp 3′ regions of nucleotide sequence identity between 3F22 donor sequences (dashed-line boxes). Nucleotide substitutions in the 3F22 duplication junction are shown in lowercase (donor A → g and donor T → a). Nucleotide numbering, after Edwards *et al.* (1990), indicates the last nucleotide of sequence identity between the junction and 5′ (top numbers) or 3′ (bottom numbers) donor sequences. (**B**) Structure of donor *HPRT* sequences (horizontal solid lines) aligned at the 3F22-HG4 crossover regions, shown as arrows (↕). The positions and orientations of *Alu* repeats (boxed arrows), of a single Donehower repeat (D), and of *HPRT* exon 2 (hatched box) are indicated. Nucleotide numbering indicates the boundaries of the junction regions in *HPRT* donor sequences. (**C**) Homology plot of nucleotide sequence similarity between donor sequences containing the 3F22 and HG4 junction regions. Donor sequences of 600 bp (5′ donor) and 700 bp (3′ donor) were aligned and compared using the UWGCG COMPARE function (Devereux *et al.*, 1984). Windows of 10 nucleotides of each donor sequence were compared at a stringency setting of 7 (i.e., a dot appears on the diagonal when 7 or more nucleotides of the aligned windows were identical). The extensive sequence identity between donor duplexes is indicated by the central diagonal, on which the locations of the 3F22 and HG4 junctions are indicated. The blocks of donor sequence similarity to the right of the diagonal are GT-rich repeats contained in *Alu* elements. Axis numbers indicate the location of donor sequences in the human *HPRT* sequence (Edwards *et al.*, 1990).

second duplication mechanism could involve the generation of *HPRT* exon 2–3 segments of different lengths as DNA replication intermediates, followed by unequal homologous or nonhomologous recombination with the *HPRT* locus to create the observed direct *HPRT* exon 2–3 duplications (see, e.g., Painter and Kapp, 1991). One prediction of this second mechanism is that an endogenous bidirectional DNA replication origin is present in the human *HPRT* exon 2–3 region. Two consensus yeast ARS core sequences are located in this region of the human *HPRT* gene (Edwards *et al.*, 1990), and another replication origin with an associated nuclear matrix attachment region (MAR) has been postulated to exist in adjacent *HPRT* intron 1 DNA (Sykes *et al.*, 1988). This model could be explored further by using one of several methods to determine whether these *HPRT* gene segments function as replication origins *in vivo* (Handeli *et al.*, 1989; Krysan and Calos, 1991; Vassilev *et al.*, 1990).

One particular advantage of the human *HPRT* gene for mechanistic analyses of duplication or other human mutation pathways is the availability of the complete locus sequence (Edwards *et al.*, 1990), a large collection of locus region-specific and flanking probes (Jolly *et al.*, 1982, 1983; Patel *et al.*, 1986; Yang *et al.*, 1988; Fukuchi

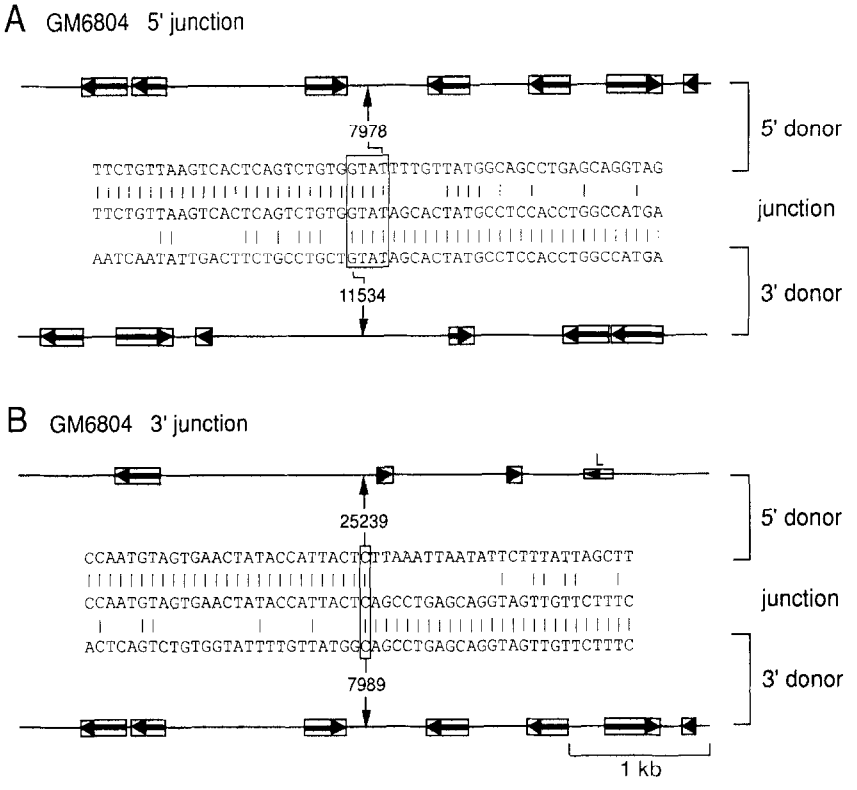

FIG. 5. Nucleotide sequence and structure of GM6804 *HPRT* gene duplication junctions. Junction sequences have been aligned with 5′ and 3′ donor nucleotide sequences and with the 5 kb of 5′ and 3′ donor *HPRT* gene sequence (horizontal solid lines) for each junction. Donor nucleotide sequences are numbered after Edwards *et al.* (1990) to indicate the last nucleotide position of sequence identity between junction and 5′ (**top**) or 3′ (**bottom**) donor sequences. The positions and orientations of *Alu* repeats and of a single LINE element (L) are shown as boxed arrows. (**A**) The GM6804 5′ junction contains a 4-bp region of nucleotide sequence identity (solid-line box) between 5′ and 3′ donors. (**B**) The GM6804 3′ junction contains a single nucleotide of sequence identity between donor sequences at the junction.

et al., 1989) and well-developed somatic cell and reverse genetic methodologies. A better understanding of the biochemistry of gene duplication and amplification may have considerable practical importance, as both of these mutation pathways play pathogenetically important roles in human genetic disease and in tumor progression (see, e.g., Endicott and Ling, 1989; Meuth, 1989, 1990; Stark *et al.*, 1989; Schwab and Amler, 1990; Cooper and Schmidtke, 1991).

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