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Use of Inverse PCR to Amplify and Sequence Breakpoints of *HPRT* Deletion and Translocation Mutations

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Deletion and translocation mutations have been shown to play a significant role in the genesis of many cancers. The *hprt* gene located at Xq26 is a frequently used marker gene in human mutational studies. In an attempt to better understand potential mutational mechanisms involved in deletions and translocations, inverse PCR (IPCR) methods to amplify and sequence the breakpoints of *hprt* mutants classified as translocations and large deletions were developed. IPCR involves the digestion of DNA with a restriction enzyme, circularization of the fragments produced, and PCR amplification around the circle with primers oriented in a direction opposite to that of conventional PCR. The use of this technique allows amplification into an unknown region, in this case through the *hprt* breakpoint into the unknown joined sequence. Through the use of this procedure, two translocation, one inversion, and two external deletion *hprt* breakpoint sequences were isolated and sequenced. The isolated IPCR products range in size from 0.4 to 1.8 kb, and were amplified from circles ranging in size from 0.6 to 7.7 kb. We have shown that inverse PCR is useful to sequence translocation and large deletion mutant breakpoints in the *hprt* gene. Environ. Mol. Mutagen. 39:22–32, 2002. © 2002 Wiley-Liss, Inc.

Key words: ***hprt*; mutation; inverse PCR**

INTRODUCTION

Cytogenetic studies have shown that tumors often have chromosomal rearrangements, that is, translocations, inversions, insertions, and large deletions [Tycko and Sklar, 1990; Rowley, 1994]. These rearrangements often involve oncogenes or tumor suppressor genes, which are activated or inactivated by these alterations, respectively [Rowley, 1994; Duro et al., 1996]. In some cases, a fusion protein is created that has unique oncogenic properties [Ben-Neriah et al., 1986; Walker et al., 1987; Golub et al., 1994]. For example, the t(9:22)(q34;q11) rearrangement creates the bcr-abl protein [Ben-Neriah et al., 1986; Walker et al., 1987]. Molecular studies of the breakpoints have revealed novel mechanisms of mutation such as illegitimate V(D)J recombinase-mediated rearrangements, especially between the T-cell–receptor (TCR) genes or immunoglobulin (Ig) genes and oncogenes (e.g., *TCRA/B* locus with the *BCR* gene, *TCRA* with *MYC*) although some involve two non-TCR/Ig genes (*SIL-TAL*) [Tsujimoto et al., 1985; Fitzgerald et al., 1991; Xia et al., 1991; Breit et al., 1993; Raimondi, 1993; Cline, 1994; Sato et al., 2001]. Other sequences have been found at the breakpoints, including AT-rich palindromes [Kurahashi et al., 2000; Edelmann et al., 2001], unstable repeat region [Wiemels et al., 2000], Alu, topoisomerase II sites [Obata et al., 1999], minisatellite core recombination [Wang et al., 1998], palindromic sequences [Ishida et al., 1998], purine/pyrimidine tract [Thandla et al., 1999], palindromic hexamer [Bhagirath et al., 1995], LINE1 insertion [Liu et al., 1997], IGH switch pentamers, and translin binding sites [Jeffs et al., 1998].

Rearrangements are also seen as mutations in genetic diseases [Bech-Hansen et al., 1987; Lehrman et al., 1987a,b; Den Dunnen et al., 1989; Yen et al., 1990; Hu et al., 1991; Capon et al., 1996; Chen et al., 1997; Amos-Landgraf et al., 1999; Edelmann et al., 1999; Potocki et al., 2000; Valero et al. 2000]. Usually these are a small percentage of mutations; however, for some genes, such as Duchenne and Becker muscular dystrophy, large deletions can be a major percentage of the mutations [Den Dunnen et al., 1989]. Sequencing studies have often led to the finding of repeated sequences

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at the breakpoints [Streisinger et al., 1966; Schmucker et al., 1996], including inverted repeats [Beauchamp et al., 2000]. Also, Alu-Alu recombination or insertion appears to be a common mechanism of rearrangement [Myerowitz et al., 1987; Muratani et al., 1991; Marcus et al., 1993; Lehrman et al., 1997a,b; Ko et al., 1998; Hunt et al., 1999; Koda et al., 2000], especially with involvement of the Alu core sequence [Harteveld et al., 1997; Hiltunen et al., 2000]. LINE elements have also been found at breakpoints [Van de Water et al., 1998]. For example, recent studies at several contiguous gene syndrome loci have found common deletions at repeated sequences [Capon et al., 1996; Chen et al., 1997; Amos-Landgraf et al., 1999; Edelmann et al., 1999; Potocki et al., 2000; Valero et al., 2000]. Other types of sequences found at breakpoints include matrix attachment sites or topoisomerase sites, B-cell switch sites, polymerase sites, chi-like elements, simple purine-pyrimidine, homopyrimidine, or AT tracts, and Z-DNA (reviewed in Rainville et al., 1995; Schmucker et al., 1996; Kehrer-Sawatzki et al., 1997; Barr et al., 1998; Ueki et al., 1998). Retroviral sequences have also been shown to be involved in intrachromosomal rearrangements on the Y chromosome [Kamp et al., 2000]

The *hprt* gene is widely used as a model gene in both in vivo studies of somatic mutation and in vitro studies of mutation [Albertini et al., 1990; O'Neill et al., 1990b]. Constitutional mutation at *hprt* also causes Lesch–Nyhan syndrome and X-linked gout [Lesch and Nyhan, 1964; Jinnah and Friedman, 2000]. The *hprt* gene has shown itself capable of capturing many mutation types and many mutational mechanisms. This includes point mutations, deletions, insertions, and translocations and such mechanisms as illegitimate V(D)J recombinase-mediated deletion [Recio et al., 1990; Fuscoe et al., 1991, 1992; Rainville et al., 1995]. Rearrangements (inversions, duplications, translocations, large deletions) make up a small percentage of *hprt* somatic mutations in normal individuals or of germinal Lesch–Nyhan mutations (~15%) [Nicklas et al., 1989; O'Neill et al., 2000]; however, they can rise to 60% of in vitro irradiated cells or in radiation-exposed individuals [Nicklas et al., 1990; O'Neill et al., 1990a; Albertini et al., 1997]. Specific deletions have also been found in exposed cells or individuals [Rainville et al., 1995; Pluth et al., 1996]. It is important to determine the breakpoints of these rearrangements because these mutations can reveal interesting new mechanisms of mutation; however, this can be a difficult proposition because of the large distances involved in deletions and inversions and the unknown partner in translocations and insertions.

Previously, we mapped linked markers around the *hprt* gene [Nicklas et al., 1991; Lippert et al., 1995a,b] and developed several methods to determine the breakpoints of *hprt* deletions and other rearrangements [Lippert et al., 1995a,b, 1997; Rainville et al., 1995; Van Houten et al., 1998; O'Neill et al., unpublished observations]. We studied the breakpoints of large deletions contained within the *hprt* gene (internal deletions) by multiple PCR, to define the breakpoint regions, followed by amplification across the breakpoint and sequencing [Rainville et al., 1995]. We developed long PCR methods to amplify across breakpoints and define *hprt* breakpoint location [Van Houten et al., 1998]. We used 3′ RACE (rapid amplification of cDNA ends) to amplify *hprt* fusion partners in large deletions [Lippert et al., 1997]. In these latter studies, we found that about 1/3 of deletions made a fusion transcript. We also used pulsed-field gel electrophoresis to determine approximate deletion size by deletion mapping and by PCR for the known linked markers [Lippert et al., 1995b]. Finally, we performed karyotypic analyses on selected mutants and found cytogenetic rearrangements involving Xq26.1, where the *hprt* gene is located (O'Neill et al., unpublished observations). Although the preceding methods could isolate and sequence the breakpoints of internal deletions and some external large deletions and translocations, we were still unable to determine the breakpoints of many rearrangements.

Inverse PCR was described in 1988 [Ochman et al., 1988; Triglia et al., 1988; Fu and Evans, 1992]. In this technique, genomic DNA from a mutant is restricted and then circularized. PCR is performed around the circle using primers from the known gene across the breakpoint into the new, unknown joined sequence of the translocation, deletion, or insertion. Sequencing of this PCR product then allows determination of the breakpoint sequence. Inverse PCR has been used to sequence breakpoints of translocations or other rearrangements [van Bakel et al., 1995; Forrester et al., 1999; Akasaka et al., 2000], to sequence unknown flanking regions [Li et al., 1999; van Heel et al., 2000], to determine insertion points of viruses [Ohshima et al., 1997; Neves et al., 1998; Tonjes et al., 1999], and to determine sequence of cDNAs [Chowers et al., 1995; Huang, 1997]. This report describes the application and adaptation of inverse PCR to the sequencing of five *hprt* gene rearrangement mutations. With the near completion of the genome project, the complete sequence and location of the new sequence joined to the *hprt* gene could be determined for each mutation.

MATERIALS AND METHODS

The mutant T-cell clones that were studied were isolated from human peripheral blood using the in vivo *hprt* cloning assay, which selects *hprt*$^-$ arising in vivo in blood, or through an in vitro *hprt* cloning assay, which selects *hprt* mutant clones after an in vitro treatment with chemicals or radiation [O'Neill et al., 1987, 1990b]. Briefly, *hprt* mutant T-lymphocytes cells are selected by their ability to grow in the purine analog 6-thioguanine in limiting dilution. These clones can be enumerated to determine a mutant frequency and grown for molecular analyses of the *hprt* mutations. The characteristics of the mutant clones chosen for study are shown in Table I. Clones were chosen for analysis that had large deletions of the *hprt* gene (missing exon fragments by Southern blot) or putative *hprt* gene rearrangements (altered *hprt* fragments on Southern blot).

Based on the Southern blot and multiplex *hprt* PCR [Gibbs et al., 1990]

TABLE I. Characteristics of *HPRT* Mutant Clones

Donor's characteristics	Mutant name	Southern blot results		Multiplex PCR results	Approximate deletion size by linked marker analysis
		PSTI	HINDIII		
Individual worked on Manhattan project (plutonium exposure)	LS535G M3	Del ex7–ex9	Del ex5–ex9	−ex7/8, −ex9	~50 kb
Father of a child with Prader–Willi syndrome	LS252 A13C3	Del ex2, ex3, ex4, ex6, ex7–ex9 ex1 larger?	Del ex2–ex3, ex4, ex5–ex9 new 5.2 kb fragment	−ex2, −ex3, −ex4, −ex5, −ex6, −ex7/8, −ex9	~570 kb
Normal individual	MF33 A4G5	Del ex4, ex6, ex7–ex9	Del ex4, ex5–ex9	−ex4, −ex5, −ex6, −ex7/8, −ex9	~1.25 Mb
Normal individual	MF38A A14C4	Del ex1	No change	−ex1	<300 kb
Normal individual, cells exposed to 300 cGy in vitro	LS323 M108	Del ex3 new 4.2-kb and new 0.9-kb fragment	Del ex2–ex3 new 5.2-kb and new 6.5-kb fragment	−ex3	NA

information, a strategy was constructed to map the breakpoint to a specific *Pst*I or *Hin*dIII fragment, upon which IPCR could be performed. To determine more closely the extent of deletion, an intronic primer flanking a restriction enzyme site was paired with several downstream primers. These PCR reactions were carried out by adding 100 ng of genomic template to a solution of 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, and 2.5 U AmpliTaq. Cycling conditions on the Perkin Elmer Cetus 480 thermocycler (Perkin Elmer Cetus Instruments, Norwalk, CT) were as follows. a 5-min denaturation at 96°C, the addition of enzyme, 33 cycles of 96°C for 30 sec, 60°C for 30 sec, and 72°C for 4 min. A wild type size PCR product from such a reaction indicated that the genomic sequence for both primers was present and that they were on the same continuous DNA fragment. However, if a product was not produced that is normally present in wild type DNA, it indicated that one or both of the primers has been deleted or that one of the primers may have been translocated such that the primer pair was no longer contained on the same DNA fragment.

Once the approximate breakpoint locations were identified, appropriate primers were designed (Oligo 4.1; National Biosciences, Plymouth, MN) to perform IPCR. The method utilized for performing the IPCR is diagrammed in Figure 1. The enzyme and primers used for each mutant are listed in Table II. The primer sequences are given in Table III. As shown in Figure 1, the primers are oriented opposite to conventional PCR primers. Genomic DNA samples (5 μg), both mutant and a wild type control, were digested overnight at 37°C using 5 μl of the appropriate restriction enzyme buffer, 37.5 μl of double-distilled water (ddH_2O), and 2.5 U of enzyme. After 4 hr, 2.5 U of additional enzyme was added. A phenol/chloroform extraction was performed and the DNA was then resuspended in 45 μl of T10E1.

Ligation was carried out overnight at room temperature at two different concentrations. The first concentration contained 1 μl of digested DNA, 80 μl T4 DNA ligase buffer, 318 μl ddH_2O, and 1 U of T4 DNA ligase. The second concentration contained 10 μl of digested DNA, 80 μl T4 DNA ligase buffer, 309 μl ddH_2O, and 1 U of T4 DNA ligase. Before ligase was added, the reaction was placed at 37°C for 2 min. This product was then also phenol/chloroform extracted, and resuspended in 20 μl of ddH_2O. Two rounds of PCR were then performed using seminested primers. The first round contained 10 μl of the mutant or wild type ligation product and appropriate blank tubes were added to a solution of 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM each primer, and 2.5 U AmpliTaq. Cycling conditions for the first round involved a 5-min 98°C denaturation to nick the circularized DNA and facilitate its amplification. Thirty-three cycles of 96°C for 30 sec, 60°C for 30 sec, and 72°C for 4 min followed. Second-round PCR used 1 μl first-round product as a template and a 96°C 5-min denaturation followed by the same 33 cycles as the first round. All PCR products were then run out on a 0.8% TBE gel, where mutant and wild type products could be compared. The sizes of the PCR products for each mutant are listed in Table II.

Reactions with a unique clean IPCR product were QIAquick spin column purified (Qiagen, Chatsworth, CA), and others were QIAquick gel extraction purified (Qiagen). These products were sequenced in the Vermont Cancer Center DNA Analysis Facility on an ABI 373.

The sequences near the breakpoints were examined for simple repeats, known sequences found at other breakpoints (Table IV), human repeats (e.g., Alu, LINE), and secondary structures (hairpins). The simple repeats at the breakpoints were examined by eye, and the known breakpoint sequences were searched using the program DM5 (University of Arizona), allowing up to one mismatch [two for V(D)J recombinase consensus], the human genome repeats using RepBase (www.girinst.org), and the secondary structure using the program Lasergene (DNA Star, Madison, WI).

RESULTS AND DISCUSSION

Table I describes five mutants that gave IPCR products. All had either a large deletion extending external to the *hprt* gene by Southern blot and/or multiplex PCR (i.e., including at least exon 1 or exon 9) or showed fragment shifts on Southern blot, indicating disruption of the gene but no deletion. These latter were likely to be translocations, although they could have been inversions or insertions. For the large deletions, deletion size was estimated by analysis of markers linked to *hprt* in Xq26.1.

Figure 1 depicts the inverse PCR method that was used to amplify the rearrangement breakpoint. We first tested this method with a wild type intron 3 *Pst*I fragment and found the appropriate size fragment (data not shown). Table II lists the primers and restriction enzymes that were utilized during the IPCR for each mutant. Figure 2 also shows PCR results with one mutant (LS535G M3) using IPCR of a *Hin*dIII fragment with *hprt* intron 5 antisense and intron 6 sense primers.

Table V lists the breakpoint sequences for the five mutants. The near completion of the genome project has allowed identification of all the genomic partners involved in the rearrangements. Of note, the complete sequence of all the non-*hprt* sequence in all the IPCR products was deter-

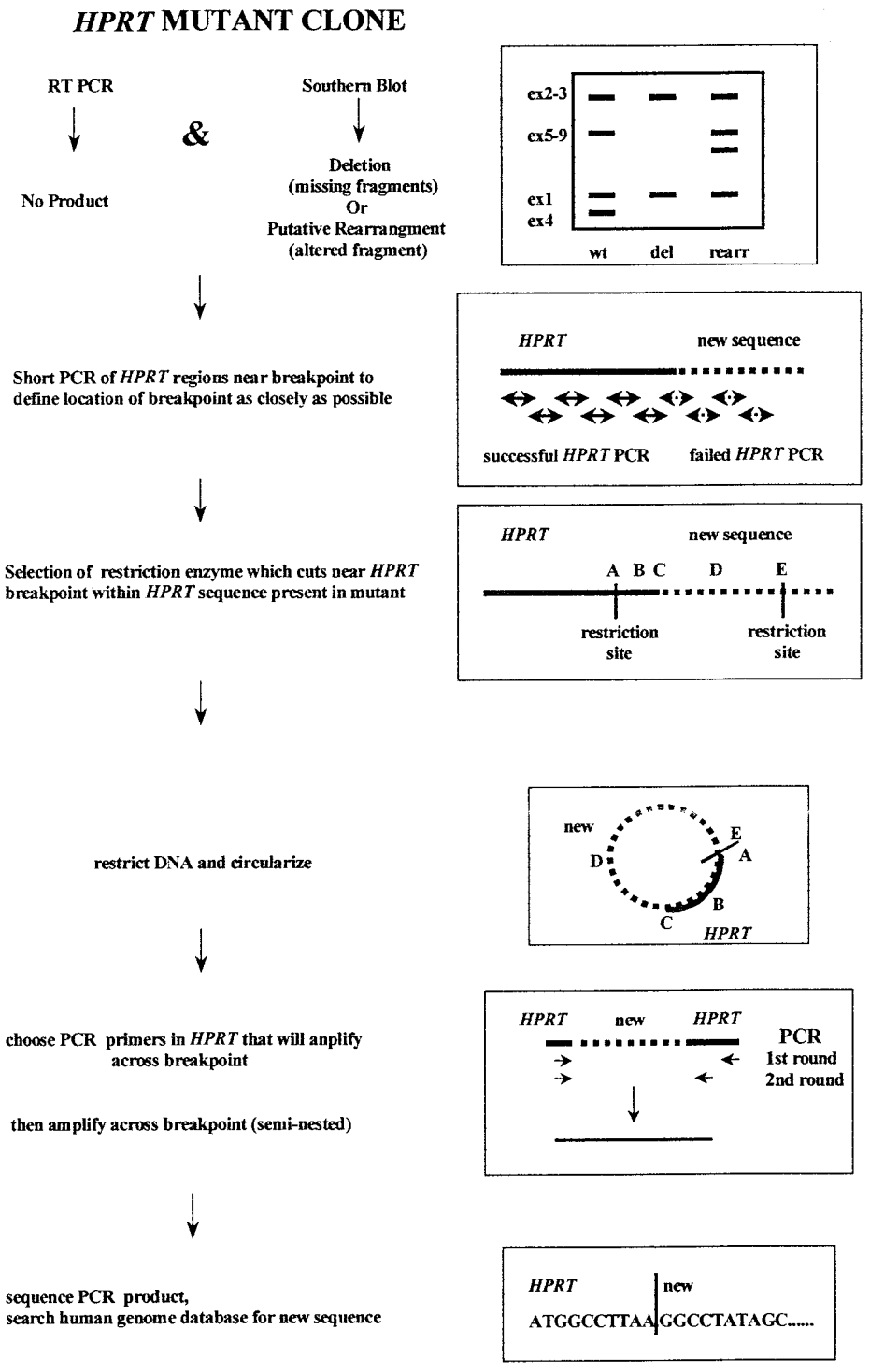

Fig. 1. Inverse PCR method.

mined including the restriction sites and corresponded perfectly to the matched sequence from the Human Genome Project; for example, the proper restriction site sequence was found in the genomic DNA for the region containing that sequence.

For the putative exon 7–9 deletion, LS535G M3, the sequence analysis shows that the mutation is a simple 22,292 basepair (bp) deletion starting in intron 6, reasonably consistent with the estimate of about 50,000 bp from linked marker analysis. The breakpoint shows no repeated sequence. Search for other possible breakpoint features showed weak consensus V(D)J recombinase sites near the

TABLE II. Inverse PCR: Restriction Enzyme for Circularization, Primers for Amplification, and Resulting PCR Product Size for Each *HPRT* Mutant Clone

Mutant name	Restriction enzyme for IPCR	IPCR circle size	PCR primers used	PCR product size	Size of "new" DNA inserted
LS535G M3	*Hin*dIII	7.7 kb	37184S	1.6 kb	712 bp
		7747 bp	32124A	1638 bp	
			31075A		
LS252 A13C3	*Pst*I	2.8 kb	11975S	1.8 kb	1060 bp
		3354 bp	11139A	2380 bp	
			11005A		
MF33 A4G5	*Pst*I	0.6 kb	24778S	0.54 kb	151 bp
		1038 bp	25434S	507 bp	
			24902A		
MF38A A14C4	*Pst*I	1.4 kb	3261S	0.4 kb	131 bp
		1364 bp	2380A	382 bp	
			2278A		
LS323 M108	*Pst*I	0.7 kb	17089S	0.4 kb	104 bp
		673 bp	17028S	393 bp	
			16808A		

TABLE III. Sequences of *HPRT* Primers Utilized

Sense primers[a]	Sequence 5′ > 3′	Antisense primers[b]	Sequence 5′ > 3′
3621	GTGGCTGTTGTTTTTATTCAGTTG	2278	TCCTTAGTTCCTTCGTGTGTCAA
11975	CTTGAATGTGATTTGAAAGGTAAT	2380	GGTAAGGACCAGATTCTCATTTTC
12879	AGACTTCTAAGAGTTTGGGTTTTC	11005	GAACTCCCTTGAAATATACACTTG
12925	GGTGATTTTTCCCCCTTACTGTGA	11139	ATGCACCATTTTGTAGTGCTTTAA
17089	CTACATCGGTTTGTGGGGAGTCAA	16808	ACCTACTGTTGCCACTAAAAAGAA
24978	GTAGAGGAGAGGGTAGAGCAACTC	17028	AAAAAGTATCCCAAGTCCCAACAG
25434	AAAAAGCCTTGGGGCAAACAGGA	24902	AGAGCGTCACTGTCAACTACATCA
30598	TGCAAATACAAGTTTGAAGACTCA	31075	CCTCTCACCATAAACCCTCACTTC
31075	CCTCTCACCATAAACCCTCACTTC	32124	GAACCACATTTTGAGAACCACTGA
37184	CATTAGCAGTCATTCTCCCTTCTC	32396	TCACTAACAGCCTCTCTCTCTCTC

[a]Numbering is from 5′ end.
[b]Numbering is from 3′ end.

TABLE IV. Sequence Motifs Searched for at Breakpoints

Motif	Sequence	Reference
Vertebrate topoisomerase II cleavage site	PNYNNCNNGYNGKTNYNY	Spitzner and Muller, 1988
DNA pol frameshift hotspot	TCCCCC CTGGCG	Kunkel, 1985
DNA pol frameshift hotspot	ACCCWR	Kunkel, 1985
DNA pol frameshift hotspot	TGGNGT ACCCCA	Kunkel, 1985
Chi and chi-like sequences	GCTGGTGG CCWCCWGC	Dewyse and Bradley, 1989
Human deletion hotspot	TGPPKM	Krawczak and Cooper, 1991
Consensus Ig switch	TGGGG	Krawczak and Cooper, 1991
V(D)J recombinase consensus site	CACWGTG	Shuman, 1991

breakpoints (Fig. 3). The *hprt* sequence had the sequence tactgtt [two bases off the consensus cac(a/t)gtg] 13 bases 3′ of the breakpoint, but no obvious nonamer (consensus acaaaaacc) either the expected 12 or 23 from the heptamer. The new sequence had two possible heptamers (caacagac and tactgtt, both two bases off the consensus) 3 and 15 bases, respectively 5′ of the breakpoint. There is a nonamer-like sequence, agcatttat 12 bases 5′ of the putative heptamer (consensus ggtttttgt).The *hprt* breakpoint is also within a LINE sequence (L1MB5) and the other breakpoint is also within a LINE element (L1MC5), although there is not alignment of the two LINE elements.

The putative exon 2–9 deletion, LS252 A13C3, has a breakpoint in intron 1 and deletion of approximately 500 kb based on the genome sequence. This is a little less than the estimate of about 570 kb from pulsed-field studies; however, there is an official gap in the Human Genome sequence between the two breakpoints that will underestimate the distance. There is a 1-bp repeat at the breakpoint. Figure 3 also shows low homology V(D)J recombinase sites near

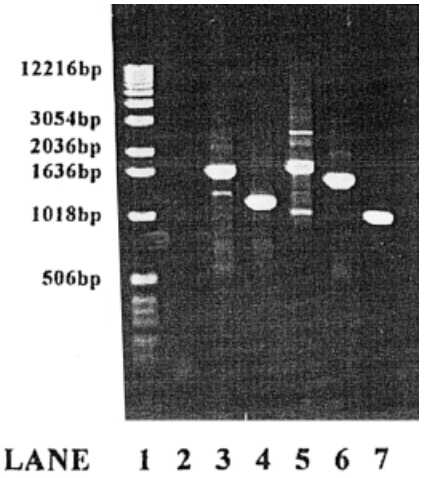

Fig. 2. PCR products from inverse PCR of *Hin*dIII cleaved and ligated DNA from mutant LS535G M3. The *hprt* breakpoint is at 37594 [Edwards et al., 1990, nomenclature]. The *Hin*dIII site is at 30555–30560. Lane 1: 1-kb ladder; lane 2: sense primer 37184, antisense primer 32124; lane 3: sense primer 37184, antisense primer 31075; lane 4: sense primer 37184, antisense primer 30598; lane 5: sense primer 37338, antisense primer 32124; lane 6: sense primer 37338, antisense primer 31075; lane 7: sense primer 37338, antisense primer 30598.

the breakpoints. tactggg is found 16 bases 3′ of the *hprt* breakpoint with no obvious nonamer, and gactgtt is 8 bases 5′ of the new sequence breakpoint, also with no obvious nonamer. The *hprt* breakpoint is also within an *Alu* sequence and about 60 bp 5′ from a series of ttttg repeats, although the other breakpoint is not. The other breakpoint sequence is in the 3′UTR of a gene, hypothetical protein (GenBank accession number AL137163, XM010423.1 mRNA).

The results for the putative exon 4–9 deletion, MF33 A4G5, were unexpected, showing *hprt* intron 3 fused to a sequence on 3q24. This indicates that rearrangement is a translocation rather than a simple deletion. Since *hprt* exons 4–9 are not present by multiplex PCR, the rearrangement must be both a translocation [probably der (Xpter-Xq26: 3q24–3qter)] and a deletion of *hprt* exons 4–9 to up to 1.25 Mb distal to *hprt* (based on pulsed-field analysis). Unfortunately, it is not possible to check the orientation of the breakpoint piece of 3q26.31with regard to the centromere, given that the genomic contig (AC004081) consists of 25 unordered pieces. The disposition and arrangement of distal Xq and proximal 3 are unknown. Given that the 3q24 proximal sequence is known, it would be possible to attempt IPCR from that sequence into what would presumably be distal Xq(27?). We did attempt IPCR from the known 3q24 sequence in wild type, nonmutant cells, before we were able to obtain the full 3q24 sequence from the Genome Project. We obtained several products; however, the one product that gave a readable sequence gave uninterpretable results (the best match was a "joining" of several short segments proximal to *hprt* on Xq, suggesting a nonspecific amplication or that the sequence is not yet in the Human Genome database). There is a 9-bp match of the sequences 3′ to the breakpoint, which must have occurred at *hprt* bp 68,739 and AC061708 bp 31,530 because the starred base in Table V matches the new sequence and not the *hprt* sequence. Recombination at this repeat match was probably the mechanism of rearrangement. There are no known repeated elements at or near either breakpoint. Both breakpoints are within 6-bp stems of stem/loop structures.

MF38A A14C4 also appeared to be a putative deletion of exon 1; however, it gave a fusion of distal *hprt* intron 1 to a sequence from 13q14. Because *hprt* exon 1 is not present by multiplex PCR and the breakpoint itself would not disrupt exon 1 amplification, the rearrangement must be both a translocation (probably der 13pter-13q14: Xq26-Xqter) and a deletion of exon 1. Unfortunately, it is not possible to check the orientation of the breakpoint piece of 13q14 with regard to the centromere because the genomic sequence (AC061708) consists of 25 unordered pieces. The disposition and arrangement of distal 13q and proximal X are unknown. Since the exon 1 deletion could be small, it might be possible to perform long PCR from 5′ *hprt* to 13q14 in preference to IPCR from distal 13q14 to trap the other breakpoint. Of interest, there was a 12-bp repeat at the breakpoint and recombination at this sequence was probably the mechanism of rearrangement. There are no known repeat elements at either breakpoint. The overlap at both breakpoints spans a stem and loop.

LS323 M108 was predicted to be a translocation because of the presence of new fragments on Southern blot. However, this mutation was also a surprise because the attached sequence was from Xq23, indicating a probable paracentric inversion involving Xq23 and Xq26. The *hprt* breakpoint occurs in the middle of exon 3 near the run of six G's, which is a hotpoint for frameshifts; this is the reason that amplification of exon 3 fails in multiplex PCR. There is a 4-bp repeat at the junction. The other breakpoint is within an MLT1A element, although the *hprt* breakpoint is not near or within any known elements. Both breakpoints are within loops of a stem/loop structure.

Thus, in the five breakpoints we found three with direct repeats at the breakpoints. The two without repeats had possible V(D)J recombinase sites and in addition LS535G M3 had LINE1 elements at both breakpoints. In our previous studies of *hprt* internal deletion breakpoint sequences [Rainville et al., 1996] we found that 10 of 21 mutations had 2- to 5-bp repeats at the breakpoints, three mutants had breakpoints at the bottom of hairpins, several occurred at consensus topisomerase sites, and one breakpoint occurred at the end of a Donehower element. We also found a cluster of breakpoints in exon 6 that occurred near a stem/loop structure. Osterholm et al. [1996] also studied16 *hprt* deletions and found that most deletions involved short repeats at the deletions and occurred in AT-rich regions. They found a 9-bp palindrome and TGA direct repeat in the 5′ region of

TABLE V. DNA Sequences of Breakpoints

Mutant name	Breakpoint sequence	*HPRT* breakpoint (~117.5 Mbp)	Other breakpoint	Comments
LS535G M3	HPRT intron 6* ACATTTTGAGGAATTGCCCGACTATTTAACAAGGTATATGTACTGTTTTACACC |||||||||||||||||||||||||||| ACATTTTGAGGAATTGCCCGACTATTTACACTGACTACTCAAATAATACATGAG |||||||||||||||||||||||||||| CATTTATACTGTTTTCGACACAGACTACCACTGACTACTCAAATAATACATGAG *AC004383	IVS6 −2221/−2220 AC004383 * = bp 80,724	Xq26 (~117.5 kbp) AC004383 * = bp 103,027	Deletes 22,292 bp
LS252 A13C3	HPRT intron 1* AACTCCTGACCTCAGGTGATACGCCCACCTGGGCCTCCCAAAATACTGGGATTA |||||||||||||||||||||||||| AACTCCTGACCTCAGGTGATACGCCCAAACCAACCTCAGGCATACATTTGTGTT |||||||||||||||||||||||||||||| TTAGAAGCAGAGACTGTTTTCTGCAAAAACCAACTCCAGGCATACATTTGTGTT *Z83826	IVS1 −1530/−1529 AC004383 * = bp 56,444	Xq26 (~117.9 Mbp) Z83826 * = bp 21,680	Deletes ~500 kbp, one basepair repeat at breakpoint
MF33 A4G5	HPRT intron 3* TCTTGAATATTTTTTCCTTTATTCCTCTTGTCTCTGTAAAGACATCAACTGGAG ||||||||||||||||||||| |||||||| TCTTGAATATTTTTTCCTTTATTCATCTTGTCTCAGAGCATCCTCATCTCTTTC |||||||||||||||||||||||||||||| AAAAAATTAGTTCACTCGGTTGGAATCTTGTCTCAGAGCATCCTCATCTCTTTC *AC061708	IVS3 −2277/−2276 AC004383 * = bp 68,739	3q22.31 (~168 Mbp) AC061708 * = bp 31,530	Probable translocation, 9-bp match at breakpoint
MF38A A14C4	*HPRT intron 1 GAAAGCGACCACCTGGGAGGGCGTGTGGGGACCAGGTTTTGCCTTTAGTTTTGC |||||||||||||||||||||||||| AATTTCCAGTTTAATACTGCATCCATGGGGACCAGGTTTTGCCTTTAGTTTTGC |||||||||||||||||||||||||||||| AATTTCCAGTTTAATACTGCATCCATGGGGACCAGGTAGAAGCACTGTTTACAT *NT 024560	IVS1 +462/+643 AC004383 * = bp 45,359	13q14 (~43.5 Mbp) NT_024560 * = bp 362,428	Probable translocation, 12-bp repeat at breakpoint
LS323 M108	*HPRT exon 3 CACATTGTAGCCCTCTGTGTGCTCAAGGGGGGCTATAAATTCTTTGCTGACCTG |||||||||||||||||||||||||| CATAAGGCATTGATTCCATTCGTGAAGGGGGGCTATAAATTCTTTGCTGACCTG |||||||||||||||||||||||||| CATAAGGCATTGATTCCATTCGTGAAGGCTCTATCCTCATGACCTTACCACCAA AC004081*	exon 3 bp 208–209 AC004383 * = bp 59,812	Xq22.1 (~89.8 Mbp) AC004081 * = bp 39,789	Probable inversion, 4-bp repeat at breakpoint

LS535G M3

gctaggtcatatagtaactctgtgtttaacattttgaggaattgcccgactattt|aacaaggtatatgtactgttttacaccagtaacatatgagggttccaatatctccacatcct

ttataaggaaactttttaataaacaagcatttatactgttttcgacacagactac|cactgactactcaaataatacatgaggggatatttctttttatggaagta

ttataaggaaactttttaataaacaagcatttatactgttttcgacacagactac|cactgactactcaaataatacatgaggggatatttctttttatggaagta

ttataaggaaactttttaataaacaagcatttatactgttttcgacacagactac|cactgactactcaaataatacatgaggggatatttctttttatggaagta

LS252 A13C3

ttcaccatgttggccaggctggtctctaactcctgacctcaggtgatacgccc|a|cctgggcctcccaaaatactgggattacaggcatgagccgctgcatcagccagcagtttttc

cctatggaagactagacatagaagtttttagaagcagagactgttttctgcaa|a|aaccaacctcaggcatacatttgtgtttacatatattccttgagaat

| breakpoint

cacwgtg - VDJ recombinase heptamer consensus

ggtttttgt or acaaaaacc- VDJ recombinase nonamer consensus

Fig. 3. V(D)J recombinase consensus sequences near the breakpoints of LS535G M3 and LS252 A13C3.

exon 2, which was involved in six of the seven exon 2 deletions.

An important concept to consider is that sequencing across the *hprt* breakpoint in the IPCR product of the mutant does not necessarily give the sequences of all the relevant genomic sequences involved in the rearrangement. For example, if the mutant is a translocation, then sequencing of the other *hprt* breakpoint will be required to determine whether bases were lost or added during the rearrangement. However, since the sequences at the reciprocal event can be inferred from the known breakpoint, it should be relatively easy to design primer and amplify this second breakpoint. Problems will arise only if large sequences were lost during the rearrangement or if multiple chromosomes were involved. We have seen several such complex mutations in cytogenetic analyses (unpublished observations).

As discussed earlier, one of the major steps in sequencing the breakpoints of mutants is determination of an approximate position of the *hprt* breakpoint. Although we used regular PCR to determine breakpoint locations for the mutants studied in this investigation, the ability to use long PCR should simplify this process. Our group recently used *hprt* long PCR methods we developed [Van Houten et al., 1998] to trap and sequence *hprt* breakpoints [Brooks et al., 2001].

There is difficulty in also obtaining products from some mutants. In addition to the mutants with successful IPCR reported here, we attempted IPCR on an additional nine mutants. Two gave products but we were unable to obtain a sufficiently clean sequence to identify the breakpoint; the others did not give a product. This failure could be because the restriction sites are such that the circle is too large or too small. Other groups have combined IPCR and long PCR to advantage [Willis et al., 1997; Akasaka et al., 2000], which we will also attempt in future studies.

In conclusion, we have shown that IPCR is a valuable technique for isolating and sequencing *hprt* rearrangement breakpoints.

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