Advances in Brief

V(D)J Recombinase-like Activity Mediates hprt Gene Deletion in Human Fetal T-Lymphocytes


Abstract

Studies from several laboratories worldwide have developed a large database for in vivo hypoxanthine-guanine phosphoribosyltransferase gene mutations in human T-lymphocytes. Sufficient differences have been found thus far between the spectrum for spontaneous mutations in adults and that observed in the fetus to suggest fundamental differences in in vivo mutagenic mechanisms at these two life stages. In adults, only ~15% of hypoxanthine-guanine phosphoribosyltransferase mutations have structural alterations on Southern blots, while in the fetus 75% of mutations show alterations of which one-half are deletions of exons 2 and 3. We have now sequenced the breakpoint sites for these specific deletions in 18 mutant lymphocyte clones isolated from 13 normal newborns. Three classes of deletions were found. Each class had the same intron 1 breakpoint but a different intron 3 breakpoint. These mutations have all the signatures of a V(D)J recombinase-mediated event (a 5' consensus heptamer, 3' consensus heptamer and nonamer, nicking, non-germline-encoded nucleotides, P-nucleotides). At the 3' breakpoint of the most common class (comprising 83% of the mutants) a perfect heptamer can be created by postulating a hairpin loop which could attain a Z-DNA configuration. This feature may indicate recombination preference for certain DNA structures. These results implicate the V(D)J recombinase in illegitimate events causing mutation in this housekeeping gene during T-cell development. Inactivation of genes involved in the control of growth and differentiation (e.g., tumor suppressor genes) by this mechanism may have important implications for cancer development.

Introduction

hprt is a constitutively expressed but nonessential gene located at Xq26 in humans. It is widely used for both in vitro and in vivo mutagenicity studies because of the ease of mutant selection with purine analogues (1, 2). In humans, hprt is 43 kilobases in length and includes nine exons (1). The genomic region including and surrounding hprt has been well characterized by sequence analysis of 57 kilobases of DNA (3). Studies from several laboratories worldwide have developed a large database for in vivo hprt mutations in human T-lymphocytes (2). Mean mutant frequencies for normal adults are 5–10 × 10⁻⁶, with elevations up to 100-fold reported for individuals exposed to mutagens. The studies are usually performed using a cloning assay (2), which permits mutant lymphocyte clones to be propagated in vitro for molecular characterization. Southern blot analyses have shown that approximately 15% of the spontaneous mutations in normal young adults are due to gross structural alterations such as deletions or insertions (2). Among these, breakpoint sites occur randomly within and beyond hprt. The remaining 85% of spontaneous mutations show simple base substitutions arising from transitions and transversions, small deletions or insertions, frameshift, and splice-site mutations (2). The sites of “point mutations” also occur randomly, as judged by their position in cDNA, and appear to arise in a distribution similar to that seen for germinal Lesch-Nyhan hprt mutations (2). Several laboratories have also reported studies of in vivo hprt mutations in the fetus, as determined from mutant lymphocyte clones isolated from umbilical cord blood (2). Mean mutant frequencies have been consistently lower than for adults, usually 10⁻⁶ or less. Two studies from our laboratory have found these to be 0.6 ± 0.4 × 10⁻⁶ (4) and 1.8 ± 2.0 × 10⁻⁶ (5), respectively. Southern blot analyses of mutants isolated in these studies (5, 6) have shown that 75% contain visible structural alterations of hprt, thus differing from findings in adults. Importantly, these deletions are predominantly intragenic, with breakpoints clustered in introns 1 and 3, deleting exons 2 and 3. We now report further characterization of 18 exon 2–3 deletion mutant clones isolated from 13 normal newborns and describe the mechanism by which these deletions arose.

Materials and Methods

Selection of Mutant Clones. All blood samples were obtained with informed consent as approved by the University of Vermont Committee on Human Research. hprt mutants from umbilical cord blood were isolated by use of a T-lymphocyte cloning assay using 6-thioguanine as the mutant selection agent (4, 5). hprt mutants were expanded in vitro, and DNA was isolated for molecular analysis (5, 6). Southern blot hybridization using a 5' PstI–XhoI fragment of hprt cDNA probe pHPT31 (1), which is specific for exons 1 and 2, was performed after PstI and HindIII digestion.

Polymerase Chain Reaction. Multiplex hprt PCR was performed as described (7). PCR (8) across the breakpoint sites was carried out using the 5' oligodeoxynucleotide 5'-CAGTTTCCCGGGTTCGG-3', which anneals at nucleotides 22718-22741. Automated thermal cycling was for 33 cycles of 94°C, 1 min; 60°C, 1 min; 68°C, 2–4 min after an initial denaturation of 94°C for 4 min. The 50-μl reaction mixture consisted of 200–300 ng DNA, each primer (1.2–1.8 μM), and 5 units Taq polymerase in buffer (described in Ref. 9) without bovine serum albumin. This primer set was used to initially screen the 18 deletion

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RESULTS AND DISCUSSION

Analysis of \textit{in vivo} fetal hprt deletion mutants by Southern blotting demonstrated that many of the mutants had hprt gene deletions which appeared to have similar (or identical) losses of exons 2 and 3. Blots of PstI digests of DNA from most of these mutants (class I) showed losses of the normal hprt exon 1, 2, and 3 fragments with the gain of a novel exon 1-containing fragment (2.3 kilobases) that was smaller than the normal exon 2-containing fragment (Fig. 1). When \textit{Hind}III digests of these deletion mutants were analyzed, the normal exons 2 and 3-containing fragment was lost, and a larger (7.4 kilobases) fragment was identified, which has been shown to contain exon 1 by using a 5' specific hprt probe (not shown). Study of the remaining exons 2 and 3 deletion mutants suggested that these mutants fell into two additional classes based on the sizes of exon 1-containing restriction fragments (Table 1).

Analysis of individual exons in the 18 mutants described in Table 1 using the multiplex polymerase chain reaction (7) verified that exons 2 and 3 were indeed deleted and that the remaining exons (including exon 1) were intact (data not shown). This confirmed that the hprt genes had undergone similar intragenic deletions in all mutants. Based on the Southern blot sizes of the common new exon 1-containing fragments in the 15 class I mutants and the position of the multiplex exon 1 primers, their exon 1 breakpoint was localized to a region of approximately 378 base pairs immediately 3' of exon 1 (nucleotides 1851–2228, according to Ref. 3) and their intron 3 breakpoint to a 379-base pair region near the midpoint of this intron (nucleotides 22099–22477).

A 5' flanking primer for the intron 1 breakpoint region and a 3' flanking primer for the intron 3 breakpoint region were then synthesized and used for PCR amplifications of genomic DNA from the 18 fetal hprt deletion mutants. Each mutant yielded a single amplified fragment. The 15 class I mutants produced a product of \approx 860 base pairs, while the two class II mutants yielded a 3000-base pair fragment and the one class III mutant gave a 570-base pair fragment. Similar treatment of DNA from wild-type T-cell clones yielded no amplification products, as would be expected since the primers are 20 kilobases apart in wild-type DNA.

Restriction enzyme sites were mapped on PCR-amplified DNA fragments from each class and compared to the wild-type sequence to further localize the breakpoint junction. Primers flanking these junctions were then used to sequence DNA amplified from each of the 18 mutants. The results, which show three breakpoint junctions corresponding to the three sizes of PCR fragments, are summarized in Fig. 2 (A–C) along with the wild-type sequences. It can be seen that each of the junctions is unique, although the breakpoints in each class are tightly clustered and result in deletions of 20 (class I), 18 (class II) or 20.4 kilobases (class III).

Inspection of these sequences revealed striking similarities with previously described DNA sequences cleaved and rejoined by V(D)J recombines. This enzyme system normally rearranges TCR genes in pre-T-cells and Ig genes in B-cells and is directed by a conserved heptamer [CAC(A/T)GTG] (10, 11).
VDJ RECOMBINASE MUTATIONS IN *hprt*

Table 1  Southern blot alterations in 18 newborn exon 2 and 3 deletion mutants

<table>
<thead>
<tr>
<th>Newborn</th>
<th>Mutant</th>
<th><em>Pst</em>I exon 1 fragment size (kilobases)</th>
<th><em>Hind</em>III exon 1 fragment size (kilobases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP66A</td>
<td>M4</td>
<td>2.3</td>
<td>ND</td>
</tr>
<tr>
<td>BP66B</td>
<td>M5</td>
<td>2.3</td>
<td>ND</td>
</tr>
<tr>
<td>LS346</td>
<td>M6</td>
<td>2.3</td>
<td>7.4</td>
</tr>
<tr>
<td>LS406</td>
<td>M8</td>
<td>2.3</td>
<td>ND</td>
</tr>
<tr>
<td>LS451</td>
<td>M2</td>
<td>2.3</td>
<td>ND</td>
</tr>
<tr>
<td>MF40a</td>
<td>M1</td>
<td>ND</td>
<td>7.4</td>
</tr>
<tr>
<td>MM30</td>
<td>M9</td>
<td>2.3</td>
<td>ND</td>
</tr>
<tr>
<td>MM31</td>
<td>M1</td>
<td>2.3</td>
<td>7.4</td>
</tr>
<tr>
<td>MM32</td>
<td>M18</td>
<td>2.3</td>
<td>7.4</td>
</tr>
<tr>
<td>MM40</td>
<td>M10</td>
<td>ND</td>
<td>7.4</td>
</tr>
<tr>
<td>MM41</td>
<td>M14</td>
<td>ND</td>
<td>7.4</td>
</tr>
<tr>
<td>SS108</td>
<td>A23B2</td>
<td>2.3</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>A22G1</td>
<td>2.3</td>
<td>ND</td>
</tr>
<tr>
<td>Class II</td>
<td></td>
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<td></td>
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<tr>
<td>LS346</td>
<td>M12</td>
<td>3.6</td>
<td>9.6</td>
</tr>
<tr>
<td>LS406</td>
<td>M7</td>
<td>3.6</td>
<td>9.6</td>
</tr>
<tr>
<td>Class III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MM29</td>
<td>M8</td>
<td>ND</td>
<td>7.2</td>
</tr>
</tbody>
</table>

* Normal *Pst*I exon 1 fragment size is 3.1 kilobases.
* Normal *Hind*III exon 1 fragment size is 7.2 kilobases.
* ND, not determined.

For normal TCR and Ig rearrangements, this consensus heptamer is separated by either 12 or 23 base pairs from a conserved T-rich nonamer element [GGTTTTTGT] (11,12). Importantly, V(D)J recombinase cleaves and rejoins the DNA by a multienzyme process which produces a distinct signature. An endonuclease activity cleaves the DNA 5' to the first C in the consensus heptamer, and it is hypothesized that a 2-base pair addition (P-nucleotides) is made that results in a four-base palindrome at the breakpoint junction (11). Nib-exonucleolytic activity which often removes a small number of nucleotides at each end (13). In addition, the V(D)J recombinase has an N-nucleotidyltransferase activity which adds nontemplated nucleotides at the initial cleavage site (nibbling) and a deoxy-nucleotidyltransferase activity which adds nontemplated nucleotides (N-nucleotides) at the breakpoint junction (11). Nibbling by the exonuclease removes P-nucleotides; thus they are only found on “unnibbled” junctions.

The DNA sequences at the deletion breakpoints in Fig. 2 show similarities to TCR and Ig gene rearrangements that extend to the specifics noted above. In the predominant class of mutants (class I; Fig. 2A), a heptamer located near the breakpoint in intron 1 differs from the V(D)J recombinase consensus heptamer by a single base (A versus G) in the position farthest from the site of cleavage. Near the breakpoint in intron 3 is the sequence GTGTGTG, which matches four bases of the consensus heptamer (TTTAGTG), separated 23 base pairs from a nonamer, TGTTTTGGT. There is also “nibbling” by the exonuclease removes P-nucleotides; thus they are only found on “unnibbled” junctions.

Two mutants, representing the second class of exons 2 and 3 deletion mutations, are shown in Fig. 2B. These mutants have the same intron 1 breakpoint, but a different intron 3 breakpoint located 2.4 kilobases upstream of the consensus heptamer (17). Certain additional features of the V(D)J recombinase system mediating these deletions. The range of nibbled bases (0–15) and added bases (0–12) is similar to that previously reported (17). Certain additional features of these *hprt* mutants, and the conditions under which they arose in vivo, also add support to our interpretation that they represent illegitimate V(D)J recombinase-mediated events. First, they arose in the fetus, where intrathymic differentiation of T-lymphocytes, with attendant TCR gene arrangement, occurs. Sixteen of the 18 *hprt* mutants showed rearranged TCR genes on Southern blots (6), indicating that, in these cells at least, β and/or γ gene rearrangements had occurred. Exon 2 and 3 deletion mutants are rarely found in adults (2) where ongoing T-cell differentiation has ceased due to thymic involution. The rare exon 2 and 3 mutants found are probably remaining from
class II mutants, and I (3%) is a class III mutant. Additional mediated by the recombinase as well. Future studies of addi-

4-9 deletions) have not been examined at the DNA sequence 

center, underlined DNA sequences indicate the conserved heptamer and nonamer 

types of mutants with altered Southern blot patterns (e.g. exon 

studies, 31 have been characterized for exon 1 restriction frag 

50% of the fetal mutations detectable by Southern blot hybrid 

V(D)J recombinase appears to be responsible for approximately 

hprt gene in fetal lymphocytes. The inappropriate activity of 

as is present in genes that are actively transcribing (18). Hprt isconstitutively active in lymphocytes, and the gene's expression 

early childhood. Second, accessibility to V(D)J recombinase 

apparently is favored by chromatin in an “open” configuration, 

which are active during fetal development would be potential 

targets for V(D)J recombinase-mediated mutations. Mutations 

leukemia in which a consensus heptamer, but no nonamer, was 

22) found a 90-kilobase V(D)J recombinase-mediated “intra-

different chromosome arm. However, two recent reports (21, 

phoblastic leukemia (21,22), and chromosomal rearrangements 

events have been implicated in several forms of leukemia, 

including chronic lymphocytic leukemia (20), T-cell acute lym-

phoblastic leukemia in which a consensus heptamer, but no nonamer, was 

found at the break sites. Our report is the first of V(D)J 

recombinase-mediated events have been implicated in several forms of leukemia, 

such early fetal 

targets for V(D)J recombinase-mediated deletions [or inversions, if, 

the orientation of the recognition sequences is inverted (17)] 

may play an important role in B- and T-cell-derived cancers 

and perhaps other disease. In fact, V(D)J recombinase-mediated 

events involve a normal substrate for V(D)J recombinase activity (an 

Ig or TCR gene) and a presumed or known oncogene on a 

different chromosome arm. However, two recent reports (21, 

22) found a 90-kilobase V(D)J recombinase-mediated “intragenic” 

deletion in the tal-1 gene of T-cell acute lymphoblastic 

leukemia in which a consensus heptamer, but no nonamer, was 

found at the break sites. Our report is the first of V(D)J 

recombinase-like activity resulting in intragenic deletion in a housekeeping gene.

Genes involved in the control of growth and differentiation which are active during fetal development would be potential 

targets for V(D)J recombinase-mediated mutations. Mutations 

in these genes and, in particular, tumor suppressor genes may lead to alterations in growth control of the cell. Such early fetal 
cell mutations have important implications for cancer devel-
opment, since early mutations have the opportunity for expan-
sion to large clones as development proceeds. This provides
more cells in which subsequent oncogenic mutation may occur
and more cell divisions during which replication errors can
occur and be fixed.

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blots, and Dr. Richard A. Gibbs for a gift of the multiplex PCR deletion
screening kits.

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