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Human heavy-chain variable region gene family nonrandomly rearranged in familial chronic lymphocytic leukemia

(DNA rearrangements/B lymphocytes/somatic mutation)

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ABSTRACT **We have identified a family of human immunoglobulin heavy-chain variable-region (V_H) genes, one member of which is rearranged in two affected members of a family in which the father and four of five siblings developed chronic lymphocytic leukemia. Cloning and sequencing of the rearranged V_H genes from leukemic lymphocytes of three affected siblings showed that two siblings had rearranged V_H genes (V_HTS1 and V_HWS1) that were 90% homologous. The corresponding germ-line gene, V_H251, was found to be part of a small (four gene) V_H gene family, which we term V_HV. The DNA sequence homology to V_HWS1 (95%) and V_HTS1 (88%) and identical restriction sites on the 5′ side of V_H confirm that rearrangement of V_H251 followed by somatic mutation produced the identical V_H gene rearrangements in the two siblings. V_HTS1 is not a functional V_H gene; a functional V_H rearrangement was found on the other chromosome of this patient. The other two siblings had different V_H gene rearrangements. All used different diversity genes. Mechanisms proposed for nonrandom selection of a single V_H gene include developmental regulation of this V_H gene rearrangement or selection of a subpopulation of B cells in which this V_H has been rearranged.**

The prevalent form of adult leukemia in western countries is chronic lymphocytic leukemia (CLL), in which the normally diverse population of B lymphocytes is replaced by a nearly monoclonal accumulation of cells (1). The disease has a tendency to cluster in families (2, 3) indicating that genetic factors may be involved in its etiology.

It is not yet known whether particular gene arrangements turn up in CLL, especially in familial cases. Variable-region gene selection could be purely random or quite biased if the disease were to directly influence DNA rearrangement or antigen driven clonal expansion. The study of CLL variable regions at the DNA sequence level may shed light on the genetic and/or environmental factors involved in the disease. We have analyzed three members of a family in which the father and four of five siblings developed CLL (4). This family has been studied by W. Blattner and coworkers (5) who showed that the cells express surface IgM and IgD with κ light chains. We have found rearrangement of the same heavy-chain variable-region (V_H) gene in the two male siblings of this family. This particular V_H gene has an unusual DNA sequence that we suggest defines a previously unrecognized V_H gene family. It is represented in the human genome by, at most, four homologous sequences.

MATERIALS AND METHODS

Frozen CLL lymphocytes obtained by leukophoresis and fibroblasts of the affected siblings were provided by W. Blattner (Environmental Epidemiology Branch, National Cancer Institute, Frederick, MD).

DNA was isolated from patient lymphocytes or fibroblasts, digested with restriction enzymes, electrophoresed on 1% agarose gels (10 μg of DNA per lane), blotted onto nitrocellulose (6), and hybridized to ^{32}P-labeled probes prepared as described (7). Hybridization was carried out at 68°C in a solution of 1 M NaCl, 50 mM sodium phosphate (pH 6.5), 2 mM EDTA, 10× Denhardt's solution, denatured, sonicated salmon sperm DNA at 100 μg/ml, and 0.5% $NaDodSO_4$. Filters were washed at 68°C with 1× SSC/0.1% $NaDodSO_4$ (V_H probes) or 0.1× SSC/0.1% $NaDodSO_4$ (J_H probes). (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin. 1 × SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0.)

Partial *Sau*3A libraries in Charon 30 (8) or complete *Hin*dIII or *Eco*RI libraries in Charon 35 (9) were constructed from patient lymphocyte DNA (6). The unamplified libraries were screened with the probe pJ_H (see Fig. 2). A Charon 35 *Hin*dIII library prepared from leukocyte DNA from an unrelated individual was screened with a 187-base-pair (bp) *Bgl* II–*Pst* I fragment covering V_HWS1 to isolate the germline V_H gene.

Restriction fragments hybridizing to the immunoglobulin heavy-chain joining-region genes (J_H) were subcloned into pUC13 (10) and sequenced by the method of Maxam and Gilbert (11).

RESULTS

Common J_H-Region Rearrangements in Leukemic Lymphocytes of CLL Family Members. Immunoglobulin gene rearrangements in family members were identified using a human J_H gene probe. This probe covering J_H2 to J_H6 (see J2J6 in Fig. 2*A*) can identify J_H rearrangements since the V_H-D_H-J_H (where D_H is the immunoglobulin heavy-chain diversity-region gene) joining leads to deletion of the intervening DNA and altered restriction enzyme sites on the 5′ side of the J_H region (12). Fig. 1*A* shows the family pedigree and pattern of J2J6 hybridization to fibroblast (germ-line) and lymphocyte (rearranged) DNA. Fibroblast DNAs digested with *Hin*dIII and *Bam*HI had a single 6.0-kbp J2J6-hybridizing band that is the expected germ-line size (14). Lymphocytes from patients HP, TS, and WS had two rearranged J_H-hybridizing bands characteristic of a monoclonal population plus various levels of the unrearranged 6.0-kbp band (presumably due to the presence of nonleukemic cells). TS and WS have common rearranged bands of ≈8.5 kbp and 2.8 kbp, whereas HP has 4.5-kbp and 3.0-kbp rearranged bands. DNA from PR, who

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Abbreviations: CLL, chronic lymphocytic leukemia; V_H, D_H, and J_H, heavy-chain variable-, diversity-, and joining-region genes; CDR, complementarity determining region; FR, framework region.

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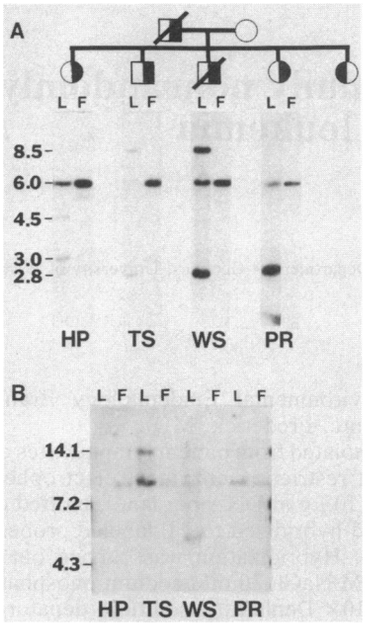

FIG. 1. Family pedigree and analysis of immunoglobulin J_H and J_κ rearrangements. ◨, Male with CLL; ◑, female with CLL; ◐, female with other neoplasm; /, deceased. (*A*) Immunoglobulin J_H rearrangements. Lymphocyte (lanes L) or fibroblast (lanes F) DNA was digested with *Hin*dIII and *Bam*HI and probed with J2J6, a 2.4 kbp *Sau*3A fragment carrying J_H2–J_H6 (see Fig. 2). Fragment sizes in kbp are indicated at the left, and patients are indicated at the bottom. (*B*) Immunoglobulin J_κ rearrangements. Lymphocyte (lanes L) or fibroblast (lanes F) DNA was digested with *Hin*dIII and probed with a 1.9-kbp *Sac* I fragment covering J_κ (13). Fragment sizes in kbp are indicated at the left and patients are indicated at the bottom.

is in spontaneous remission, showed a germ-line band and a single 2.9-kbp rearranged band. *Eco*RI digests also showed that TS and WS shared identical rearrangements (data not shown).

Since all the siblings expressed surface κ light chain, a similar analysis was carried out with a κ light-chain probe (13). Fig. 1*B* shows no common light-chain rearrangements in this family.

To determine if the common J_H-hybridizing bands were, in fact, due to identical V_H-D_H-J_H rearrangements, the rearranged genes were cloned and the V_H-D_H-J_H rearrangements were sequenced.

The 8.5- and 2.8-kbp J_H-hybridizing bands seen in Fig. 1*A* were cloned in separate phages and represent the two rearranged chromosomes (designated 1 and 2) of TS and WS. The 3.0-kbp J_H-hybridizing band of HP corresponds to the phage clones designated HP1. Fig. 2*A* shows the restriction maps of the common 8.5-kbp TS1 and WS1 rearrangements together with the corresponding germ-line J_H and V_H restriction maps whereas Fig. 2*B* shows the other rearrangements.

A Single Germ-Line Gene from a Previously Unidentified Low-Copy-Number Human V_H Gene Family is Rearranged in Both TS and WS. Probes specific for the V_H-coding region or the 5′-flanking region of WS1 and TS1 were hybridized to *Hin*dIII digests or *Hin*dIII/*Bam*HI double digests of patient lymphocyte or fibroblast DNA or of normal liver DNA. No differences in hybridization patterns were seen with the various probes. All hybridized strongly to the rearranged 8.5-kbp *Hin*dIII–*Bam*HI fragment in TS and WS lymphocytes (data not shown) and to 7.5-, 3.2-, 1.5-, and 1.4-kbp germ-line *Hin*dIII–*Bam*HI bands (Fig. 3*A*). *Hin*dIII digests showed two strongly hybridizing germ-line bands (10.5 and 5.5 kbp). (Fig. 3*B*); the 10.5-kbp band in TS was replaced by a 11.2-kbp rearranged band. The 10.5- and 5.5-kbp *Hin*dIII bands were not polymorphic among unrelated DNAs. Depending on the enzyme used, only three or four bands were ever detected, indicating that V_HTS1 and V_HWS1 were derived from a small family of V_H genes.

Germ-line clones representing the four *Hin*dIII–*Bam*HI bands of Fig. 3*A* were isolated and sequenced from DNA of unrelated individuals. The phage clones corresponding to the most strongly hybridizing 7.5-kbp *Hin*dIII–*Bam*HI fragment are shown in Fig. 2*A*; the V_H gene is called V_H251. The phages carrying V_H251, V_HWS1, and V_HTS1 have nearly identical restriction maps on the 5′ side of the V_H region, suggesting that V_HWS1 and V_HTS1 arose from rearrangement of V_H251.

The comparison of the DNA and amino acid sequences of the WS1 and TS1 V_H-D_H-J_H genes with that of the germ-line V_H251 and J_H4 genes is shown in Fig. 4. Both TS1 and WS1 used J_H4 and the same V_H segments but different D_H segments. V_HWS1 was productively rearranged. Both V_HWS1 and the germ-line V_H251 had all the features characteristic of a functional V_H gene—a conserved octanucleotide sequence ATGCAAAT located 105 bp before the translation initiation codon (16), a leader sequence with appropriate junctions for splicing to the V_H (17), and conserved residues (e.g., cysteines at positions 22 and 92) at anticipated positions (15). V_HTS1 was 90% homologous to V_HWS1 but cannot be expressed. It has terminators at amino acids 46 and 83, a thymidine to adenosine base change that eliminates the translation initiation codon at position −19, and a frame shift at the D_H-J_H junction that alters the J_H4 reading frame.

The DNA sequence homology of the germ-line V_H251 gene to V_HWS1 (95%) and V_HTS1 (88%) and identical 5′-restriction sites confirm that V_H251 was indeed the precursor to both V_HWS1 and V_HTS1. The other germ-line V_H genes are less homologous (82% and 71%, respectively, with V_HWS1) and the 5′-restriction sites do not align with those of the V_HTS1 or V_HWS1 genes (data not shown).

V_HWS1 differs by 21 bp from the germ-line gene. Substitutions were most frequent in complementarity determining regions (CDRs), with 3 base changes in CDR1 and 4 in CDR2. The proportion of replacement substitutions (those producing an amino acid change) ranges from 0.3 to 0.5 in the framework region (FR); in the CDRs the replacement proportion is 1.0. V_HTS1 had the most amino acid changes in CDR1 (4/5), CDR2 (4/17), and FR3 (10/32). The proportion of replacement substitutions in the CDRs was 1.0 and ranged from 0.5 in FR2 to 1 in FR3. A few of the observed changes, especially those in which V_HTS1 and V_HWS1 were the same but different from the germ line, could reflect the fact that the germ-line DNA came from an unrelated individual.

The germ-line sequence appears to constitute a previously unidentified V_H gene family that we propose to designate V_HV. Although its 83-bp intron size is consistent with that of the V_HI family, its closest relatives in the GenBank,¶ HG3 (18) and LR-35, are only 64% and 62% homologous. The two D_H segments are also different from any reported.

Analysis of the Other J_H-Region Rearrangements. The other chromosome of TS, TS2, carried a productively rearranged V_H gene (V_HTS2) (Fig. 5). This was a V_HII gene by its 82% DNA sequence homology to another human V_HII gene (20) and amino acid homology to other human V_HII proteins, WAH (21) and Ou (22). The second chromosome of WS, WS2, carried an aberrant rearrangement in which the 3′ end of the V_H gene and the D_H and J_H4 segments had been deleted and the coding sequence for the first 40 amino acids of the V_H gene was joined to the J_H4–J_H5 intron (data not shown).

Recall that TS and WS also shared a commonly migrating 2.8-kbp J_H-hybridizing band (Fig. 1*A*). Sequence analysis

¶EMBL/GenBank Genetic Sequence Database (1986) GenBank (Bolt, Beranek, and Newman Laboratories, Cambridge, MA), Tape Release 42.0.

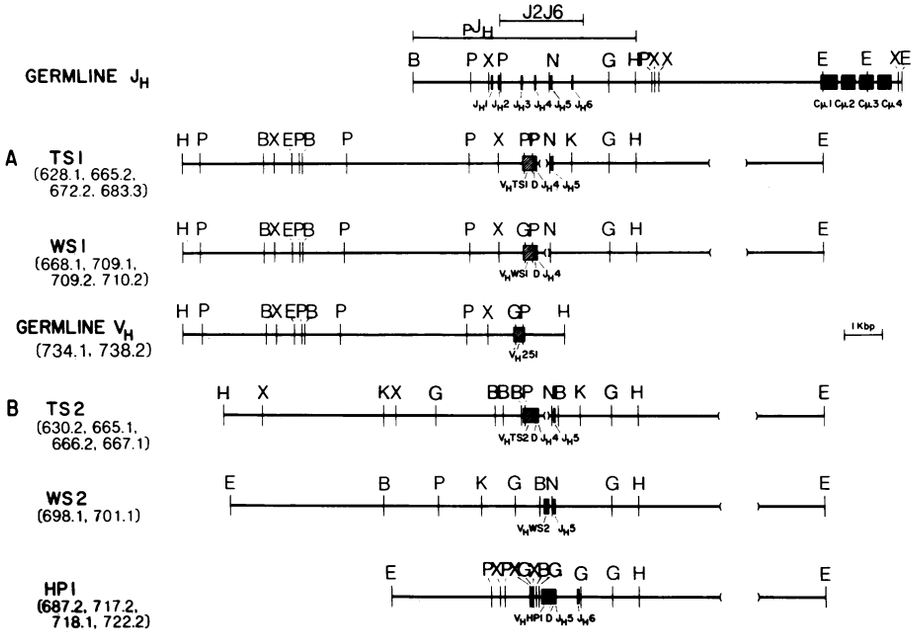

FIG. 2. Restriction maps of cloned V_H-D_H-J_H rearranged genes and corresponding germ-line V_H251 gene. ▨, V_H genes; □, D_H genes; ■, J_H and μ-chain constant-region (C_μ) genes. Parentheses indicate deletions relative to the published J_H–C_μ restriction map (14). The germ-line J_H map (14) and restriction fragments used for probes are shown above the rearranged genes. Phage clones covering the rearranged genes are indicated at the left. E, *Eco*RI; H, *Hin*dIII; Bam, *Bam*HI; P, *Pst* I; X, *Xba* I; G, *Bgl* II; K, *Kpn* I; N, *Nar* I. (*A*) Restriction maps of rearranged TS1 and WS1 chromosomes carrying the common 8.5-kbp *Hin*dIII–*Bam*HI J_H rearrangement and corresponding germ-line V_H251 gene. (*B*) Restriction maps of the other cloned J_H rearrangements. TS2 and WS2 carry the 2.8-kbp *Hin*dIII–*Bam*HI fragments of TS and WS, respectively. HP1 carries the 3.0-kbp *Hin*dIII–*Bam*HI fragment of patient HP.

revealed that this was not due to a common rearrangement, but to the following two separate events: a mutation in the J_H4–J_H5 intron creating a *Bam*HI site in TS2 and a rearrangement coupled with deletions in WS2.

Sequencing of HP1 phage clones, carrying the 3.0-kbp *Hin*dIII–*Bam*HI J_H-hybridizing band, showed a productively rearranged V_H-D_H-J_H5 (Fig. 5). The DNA sequence of V_HHP1 was 80% homologous to the V_HI genes HA2 and HG3 (18).

We did not clone the 4.5-kbp *Hin*dIII–*Bam*HI J_H-hybridizing band of HP. V_HWS1 did not cross-hybridize to this 4.5-kbp band. Hybridization of V_H251 to HP lymphocyte DNA showed only germ-line bands, indicating that V_H251 was not rearranged to any detectable extent in HP (Fig. 3*B*). Hybridization of V_HWS1 to lymphocyte DNA of PR also showed no detectable rearrangement (data not shown). The 2.9-kbp rearranged band of PR was not further characterized.

Base Changes and Deletions in the J_H4–J_H5 Intron Accompany Rearrangement. The DNA sequences of J_H4 and the J_H4–J_H5 intron in TS1, WS1, and TS2 vary considerably from the published germ-line sequence of this region (14). The J_H4–J_H5 introns of TS1 and WS1 contain significant deletions of 280 bp and 70 bp, respectively. Two independently isolated TS1 phages had identical deletions in this region. The deletion in TS1 leading to a 8.2-kbp, rather than a 8.5-kbp, J_H-hybridizing band has been confirmed by a genomic Southern blot (data not shown). The *Kpn* I sites in TS2 and TS1 and *Bam*HI site in TS2 were found in lymphocyte DNA only, not in fibroblasts from TS. These differences do not appear to result from polymorphism, but more probably reflect somatic mutation, perhaps during the rearrangement process.

We did not find evidence for translocations into the J_H region as has been reported in two other CLL cases (23, 24). A region some 10 kbp on the 5′ side of WS1 did hybridize to multiple bands on a genomic Southern blot (data not shown); however, preliminary analysis suggests it was another V_H gene.

DISCUSSION

We have isolated and sequenced the immunoglobulin heavy-chain V_H-D_H-J_H gene rearrangements in three siblings with CLL and found that two siblings, TS and WS, had rearranged the same germ-line V_H gene (V_H251) in their leukemic lymphocytes, the former nonproductively and the latter productively. V_H251 was shown to be a single-copy gene belonging to a small, previously unrecognized, gene family we term V_HV. A third sibling, HP, rearranged a different V_H gene. All the rearrangements used different D_H segments. Random rearrangement to a single-copy V_HV gene in two individuals should be rare (frequency < 0.003, assuming the total number of human V_H genes is 200; ref. 19 and J. Berman and F. Alt, personal communication).

Preferential V_H-gene rearrangement occurs in mice where the V_H7183 family, inferred to be closest to the D_H cluster, is frequently used in the earliest B cells (25, 26). Most clones of this type are eliminated subsequently from the B-cell population. The biased rearrangement of V_H251 may be explained on a similar basis if this V_HV gene is close to D_H on the human chromosome, since CLL cells represent an early stage of B-cell development.

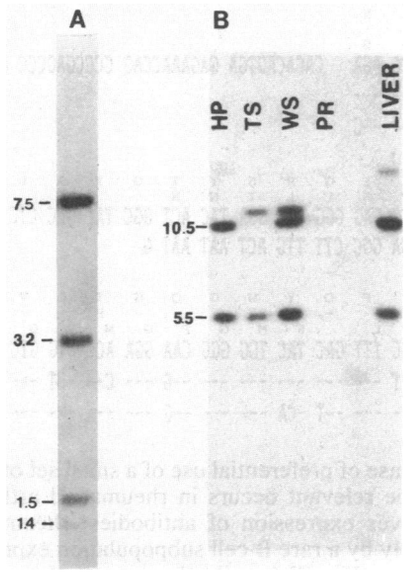

FIG. 3. Southern blot analysis of germ-line V_H251 genes. Lymphocyte DNA from the four affected siblings (indicated by initials) or normal liver DNA was digested and probed with a 1-kbp *Stu* I–*Xba* I fragment carrying the V_H251 gene and its 5′-flanking region. Fragment sizes in kbp are indicated. (*A*) Normal liver DNA digested with *Hin*dIII and *Bam*HI. (*B*) Patient lymphocyte DNA and normal liver DNA digested with *Hin*dIII.

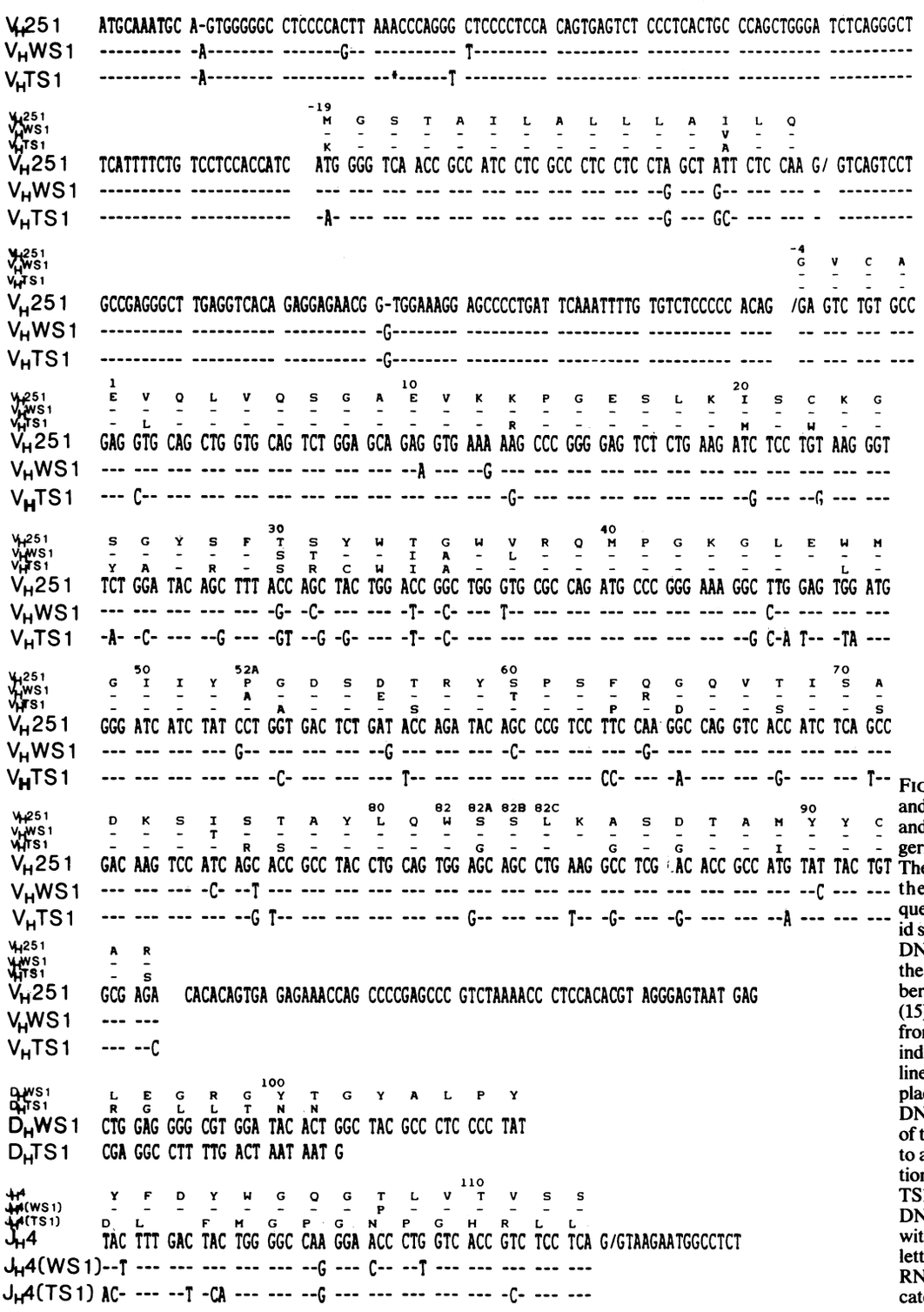

FIG. 4. Comparison of the DNA and amino acid sequences of WS1 and TS1 V_H-D_H-J_H genes with the germ-line V_H251 and J_H4 genes. The DNA sequences begin with the conserved octamer sequences. The predicted amino acid sequences are shown above the DNA sequences and are placed at the center of each triplet. Numbering is according to Kabat *et al.* (15). The J_H4 sequence is taken from Ravetch *et al.* (14). Dashes indicate identity with the germline sequence. Gaps have been placed at position 12 of the V_H251 DNA sequence (–) and position 33 of the V_HTS1 DNA sequences (*) to align the sequences. The insertion at the D_H–J_H junction of the TS1 gene is shown, and the J_H4 DNA sequence of TS1 is aligned with the germ-line J_H4. Single-letter amino acid code is used. RNA splicing positions are indicated by a slash.

Another case of preferential use of a small set of V_H genes that might be relevant occurs in rheumatoid arthritis (27), which involves expression of antibodies (rheumatoid factors), possibly by a rare B-cell subpopulation expressing the antigen Leu 1 (28). CLL cells also express Leu 1 (1). The underlying basis of rheumatoid arthritis is, of course, unknown.

Other ways to account for nonrandom V_H251 gene utilization involve selection by antigens, T-cells, or virus interacting with cell-surface V_H251 protein. This is difficult to explain in the case of the nonproductively rearranged V_H251 gene of TS unless the gene had at one time been expressed, the cells selected, and the gene later inactivated by somatic mutation. In V_HWS1 the mutations were clustered in the CDRs with a high ratio of replacement to silent substitutions. In the FR, this ratio was low. This pattern was observed in the course of an antigen response (29) and indicates conservation of framework functions and selection for receptor function at the protein level. TS1 showed less clustering in CDRs and a very high proportion of replacements throughout, which is not consistent with receptor function.

In this connection, Cleary *et al.* (30) have reported rapid accumulation of somatic mutations in the V_H region of a human B-cell lymphoma during the course of therepautic

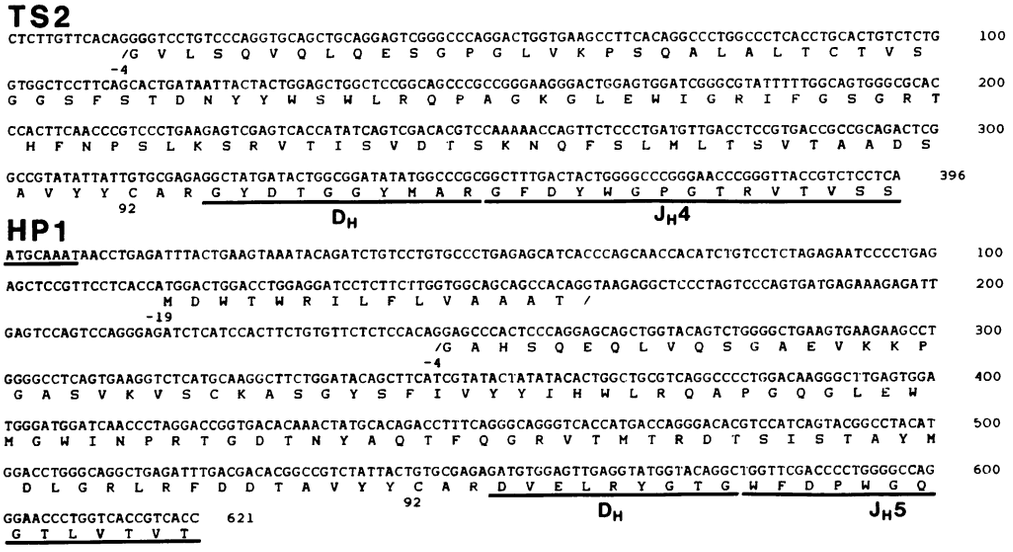

FIG. 5. Sequences of HP1 and TS2 V_H-D_H-J_H genes. The predicted amino acids are shown below the DNA sequence and are abbreviated as in Fig. 4. The octamer, D_H, and J_H sequences are underlined.

anti-idiotype treatment leading to elimination of the targeted V_H idiotope, which they interpreted as selection against cells expressing the idiotope. Although TS and WS did not receive anti-idiotype therapy, the occurrence of mutations over a decade in response to the host defenses could follow a similar pattern.

A correlation may also be drawn between expression of V_H251 and the severity of the disease. WS, with the expressed V_H251 gene and an aberrant rearrangement on the other chromosome, had the most aggressive progression of disease; whereas TS, with an unexpressed V_H251, showed a change to a more "benign" course (5). The other siblings, in whom we could detect no V_H251 rearrangement, required no therapy or exhibited spontaneous remission. If the course of the disease in this family is related to clonal stimulation of V_H251-bearing cells through either a (neoplastic) T cell or virus that recognizes its associated idiotype, the inability to prevent V_H251 expression could lead to more severe disease. This predicts that passive immunization with V_H251 or V_HWS1 antibody could block stimulation of V_HWS1-bearing cells and reduce B-cell proliferation.

Few CLL DNAs have been analyzed at the molecular level. Thus we cannot be sure whether the bias seen here is connected to this particular family or to CLL in general. We do know, however, that the V_H251 gene is not unique to this family, as it has been seen in DNA of over 100 unrelated individuals (data not shown). Now that the V_HV family of genes has been identified and characterized, the probe can be used on a large number of cases to establish its relationship to the disease in general.

In summary, we have shown that a single V_H gene belonging to a previously unidentified V_H gene family was rearranged in two siblings with CLL. More information on the relationship of this V_H gene family to CLL will depend on determining the distribution of V_H251 expression in B cells of members of this family and other CLL cases.

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