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Immunoglobulin D Switching Can Occur through Homologous Recombination in Human B Cells

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Prototypical class switching in mouse and human immunoglobulin heavy chains occurs through recombination of tandem blocks of short repeats located 5′ to each heavy chain constant region (C_H) except C_δ. Deletion of C_μ in immunoglobulin D (IgD)-secreting murine plasmacytomas occurs illegitimately. We demonstrate here that in human IgD-secreting myeloma cells freshly isolated from patient bone marrow and in normal peripheral blood B lymphocytes, an IgD switch can occur through homologous recombination of a direct repeat consisting of a 442-bp sequence 1.5 kbp 3′ of the J_H complex and a 443-bp sequence that is duplicated almost perfectly (96% similarity) 1.7 kbp 5′ of the C_δ gene (442/443-base-pair [bp] repeat). This homologous recombination mechanism is not exclusive for IgD switching, since C_μ deletion endpoints in two established IgD-secreting myeloma cell lines fall outside the 442/443-bp repeat. The 442/443-bp mediated recombination shows cell type specificity, and we propose that it represents a unique mode for increased levels of IgD secretion in humans.

The immunoglobulin heavy chain locus is located on chromosome 14 in humans. A heavy chain variable region (V_H) gene is composed of one member from each of three gene families designated variable (V), diversity (D), and joining (J). The heavy chain constant region (C_H) genes are located downstream of these V_H genes in the order C_μ-C_δ-$C_{\gamma 3}$-$C_{\gamma 1}$-$C_{\psi\varepsilon}$-$C_{\alpha 1}$-$C_{\psi\gamma}$-$C_{\gamma 2}$-$C_{\gamma 4}$-C_ε-$C_{\alpha 2}$ (3, 12, 25). In the primary immune response, the V_H gene is transcribed along with the nearest downstream constant region gene, C_μ, to form a μ heavy chain mRNA (50). During further B-cell differentiation, however, a given B cell and its progeny may shift from the production of immunoglobulin M (IgM) to one of the other heavy chain isotypes by the expression of downstream C_H genes (reviewed in references 28 and 53). Studies primarily with myelomas have indicated that the switch phenomenon is most often the result of an intrachromosomal rearrangement between switch site (S) sequences located upstream of the heavy chain genes. Upon switching, the assembled VDJ gene is brought adjacent to the heavy chain gene to be expressed, resulting in the deletion of the intervening C_H genes. Studies, at the DNA sequence level, of mice and humans have demonstrated that these S regions consist of 2 to 10 kilobases (kb) of tandem arrays of repeated sequences (8, 12, 20, 34–36, 41, 42, 48, 56–58). This description of the heavy chain class switch applies to all antibody classes except IgD. To date, the only biological function attributed to IgD with any certainty is serving as an antigen receptor along with IgM on the surface of virgin B cells (22, 26, 27, 33). After antigenic stimulation, membrane IgD disappears and is not secreted from the B cell into the serum, as is the case for IgM and the other heavy chain classes (5). It was previously determined that no DNA sequences resembling switch sites exist upstream of the mouse C_δ gene (13, 44). However, humans demonstrate a higher serum IgD content (2) and a higher occurrence of IgD myelomas (11) than do mice, suggesting that a switchlike mechanism may occur at the C_δ gene in humans. Indeed, after obtaining the sequence of the entire 19,800-base-pair (bp) region that spans the C_μ and C_δ genes in the human genome (31, 60, 61), two regions within the C_μ-C_δ intron emerged as candidates for switch recombination. First, we noted a 2-kb region containing an unusually high concentration of the pentamer TGGGG (bases 5950 to 7950 [61]) that is characteristic of all murine switch regions. Second, we (61) and others (1) noted that a 443-bp stretch about 1.7 kbp upstream of $C_{\delta 1}$ (bases 8572 to 9014 [61]) is almost perfectly duplicated (96% match) within the J_H-C_μ intron some 1.5 kbp 5′ of S_μ (30).

To determine whether either of these sequences is implicated in the secretion of IgD, we have examined primary IgD-secreting myelomas from bone marrow, two established IgD-secreting myeloma cell lines, and normal B lymphocytes from peripheral blood. We found evidence for homologous recombination of the repeat consisting of a 442-bp sequence 1.5 kbp 3′ of the J_H complex and the 443-bp stretch described above (the 442/443-bp repeat) in both normal and malignant cells and suggest that this mechanism has evolved as a unique mode for IgD expression in humans.

MATERIALS AND METHODS

Cells and cell lines. Two primary IgD-secreting myeloma samples were obtained by bone marrow aspiration from two patients (designated patient 1 and patient 2) who reported to the University of Wisconsin Hospital with serum IgD levels of approximately 20 mg/ml. Both isolates contained more than 95% tumor cells on the basis of morphology and gave prototypic myeloma phenotypes on analysis by fluorescence-activated cell sorting and radioimmunoassay. D64223 (61; J. Kearney, personal communication) and ODA (54) are IgD-secreting myelomas immortalized by fusion or Epstein-Barr virus transformation, respectively. Peripheral mononuclear cells from adult blood were enriched for B cells by the method of Jelinek et al. (18). Adult splenic lymphocytes (which were 15% B cells) were used unfractionated.

Genomic library construction, screening, and DNA sequencing. DNA was isolated from frozen cells according to the method of Blin and Stafford (4). Genomic libraries for patients 1 and 2 were constructed by partially digesting the DNA with the restriction enzymes *Mbo*I and *Bgl*II, respec-

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tively, phosphatasing the resulting partial fragments, and cloning into the bacteriophage vector Charon 40 (9). The libraries were initially screened with a nick translated, 531-bp *Sst*I-*Bgl*II fragment containing the $C_{\delta 1}$ exon (59). The resulting C_δ-positive clones were rescreened with a nick translated, 782-bp *Bgl*II-*Hin*dIII fragment near the J_H cluster (43) to isolate clones containing C_δ as well as upstream sequences.

Phage DNA fragments containing the expressed VDJ region from patient 1 and the C_μ deletion endpoints from each patient were subcloned into the mp18 and mp19 series of M13 sequencing vectors (37). The polymerase chain reaction (PCR) amplification products to be sequenced were digested with *Bam*HI after excision from agarose gels and cloned into M13mp18. The resulting DNA inserts were sequenced on both strands by the Sanger and Coulson dideoxy-chain termination method (49) with a Sequenase kit according to the protocol of the suppliers (U.S. Biochemical Corp.).

PCR. The PCR technique (47) was used to amplify the C_μ deletion (IgD switch) endpoints and the germ line (unrearranged) 442- and 443-bp regions from myeloma DNA, B-cell-enriched and -unenriched peripheral blood lymphocyte (PBL) DNA, and splenocyte and fibroblast DNA. Oligonucleotides prepared on an ABI/380A DNA synthesizer and purified by high-pressure liquid chromatography were the following: primer 1, (5′) AATGGATCCGTTCACAATCT TTTTAGGTTAACTCG (3′) (bases 515 to 540, Mills et al. [30]); primer 2, (5′) AAGGGATCCGGGAGTGACCCAGA CAATGGTC (3′) (bases 1143 to 1164, Mills et al. [30]); primer 3, (5′) ATCGGATCCCTACCTCACACATTACCCT ATCCG (3′) (bases 8503 to 8526, Word et al. [61]); primer 4, (5′) CAAGGATCCCCAGCAAACCAACAAAAGCCAGG (3′) (bases 9206 to 9232, Word et al. [61]). The underlined nucleotides represent the *Bam*HI cloning linkers added at the 5′ end of each primer to facilitate cloning. Either 0.1 ng of phage DNA or 2 μg of genomic DNA was used as a template. The PCRs were performed in a solution containing 67 mM Tris hydrochloride, 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 6.7 μM disodium EDTA, 10% dimethyl sulfoxide, 200 μM of the four deoxynucleoside triphosphates, 200 ng of each primer, and 2 U of *Taq* polymerase (6; Perkin Elmer Cetus). These reactions were performed in 50 μl of solution with 40 to 50 cycles of a programmable thermocycler. One cycle was 1 min at 90°C for denaturation and 2 min at 65°C for polymerization. A sample of PCR mixture (5 to 10 μl) was used for electrophoresis and hybridization. The resulting PCR products were centrifuged through a Sephadex G-25 Quick Spin column (Boehringer Mannheim Biochemicals) to eliminate unincorporated precursors. One half of each mixture was treated with 10 U of mung bean nuclease (MBN) (Bethesda Research Laboratories) at 37°C for 60 min. One microgram of M13 single-stranded DNA was added to each reaction to demonstrate the extent of the MBN treatment. The untreated and MBN-treated PCR mixture (10 μl of each) were used for subsequent electrophoresis.

Southern blotting. Isolated DNA was digested with the appropriate restriction enzyme(s) and electrophoresed on 0.8% agarose gels. PCR products were electrophoresed on 2% low-melting-point agarose gels. The electrophoresed DNA was transferred to GeneScreen Plus nylon membranes (Dupont, NEN Research Products) by using the Southern technique (55). The filters were prehybridized at 68°C in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate–5× Denhardt solution (0.1% Ficoll, 0.1% polyvinylpyrrolidine, 0.1% bovine serum albumin)–100 μg of denatured salmon sperm DNA per ml for 1 to 2 h. After prehybridization, filters were hybridized to isolated DNA fragments labeled with ^{32}P by the nick-translation method (45) for 16 to 20 h at 68°C in 3× SSC–0.01 M disodium EDTA–5× Denhardt solution–100 μg of salmon sperm DNA per ml. Filters were washed two times in 2× SSC at 25°C for 5 min, two times in 2× SSC–1.0% sodium dodecyl sulfate at 68°C for 30 min, and two times in 0.1% SSC at 25°C for 30 min and subjected to autoradiography.

RESULTS

The C_μ gene is deleted in two patients with IgD myeloma. A clone (designated 10.7) that was isolated from the genomic library of patient 1 was analyzed by using both restriction enzyme and Southern blotting techniques (data not shown). Hybridization experiments using probes containing C_μ sequences demonstrated that the C_μ gene was deleted from this clone. Probes containing different regions of the C_μ-C_δ intron were used in additional Southern blot analyses of clone 10.7. These experiments demonstrated that the 3′ deletion endpoint of the C_μ gene is ~2 kb upstream of the C_δ gene (Fig. 1). Identical restriction enzyme and Southern blotting experiments were used to analyze a clone (designated 16.1) isolated from a genomic library of patient 2. As in clone 10.7 from patient 1, clone 16.1 contained no C_μ gene sequences, and the 3′ deletion endpoint of C_μ occurred ~2 kb upstream of the C_δ gene. Because the 3′ deletion endpoints of clones from both IgD myeloma patients occurred over 1 kbp downstream of the TGGGG-rich region (the classical switchlike pentamer repeats), we concluded that they are not involved in the deletion of C_μ.

A 442/443-bp repeat is the site of the C_μ gene deletion in two primary IgD myelomas. The mapping data from clones 10.7 and 16.1 indicated that the 3′ C_μ gene deletion endpoint was near the copy of the 442/443-bp repeat that exists upstream of the C_δ gene. By analyzing the DNA sequence across the deletion endpoints, it was determined that the C_μ gene was deleted in these two clones via the 442/443-bp repeat and that exactly one copy of this repeat was retained in each of these clones. Figure 1 shows the configuration of the J_H-C_μ-C_δ region in the germ line and in the clones from the IgD myeloma patients.

To demonstrate that the C_μ gene deletion did not occur during the cloning process but actually exists in the genome of the IgD myeloma cells, we performed a PCR experiment. Such an experiment using primers 1 (located 5′ of the upstream 443-bp region) and 4 (located 3′ of the downstream 442-bp region) on genomic DNA from these patients should result in the amplification of an 806-bp DNA fragment if the C_μ gene deletion described above exists in the genome (Fig. 2A). Electrophoresis of products from this PCR experiment resulted in an observable fragment amplified from patient 1 and patient 2 genomic DNA that is exactly the size of the fragment amplified from 10.7 DNA (806 bp) (Fig. 2B). The authenticity of this fragment was subsequently demonstrated by Southern blotting (Fig. 2C). The probe from the 443-bp region hybridized to an additional smaller fragment as well as to the predicted 806-bp fragment (Fig. 2C). Single-stranded DNA, often generated during the PCR process (14) is degradable by MBN (23). Samples treated with MBN contained only the double-stranded predicted fragment (Fig. 2C). The additional fragment that hybridized with the probe was a single-stranded artifact of the PCR amplification.

A functionally rearranged V_H gene exists upstream of C_δ in

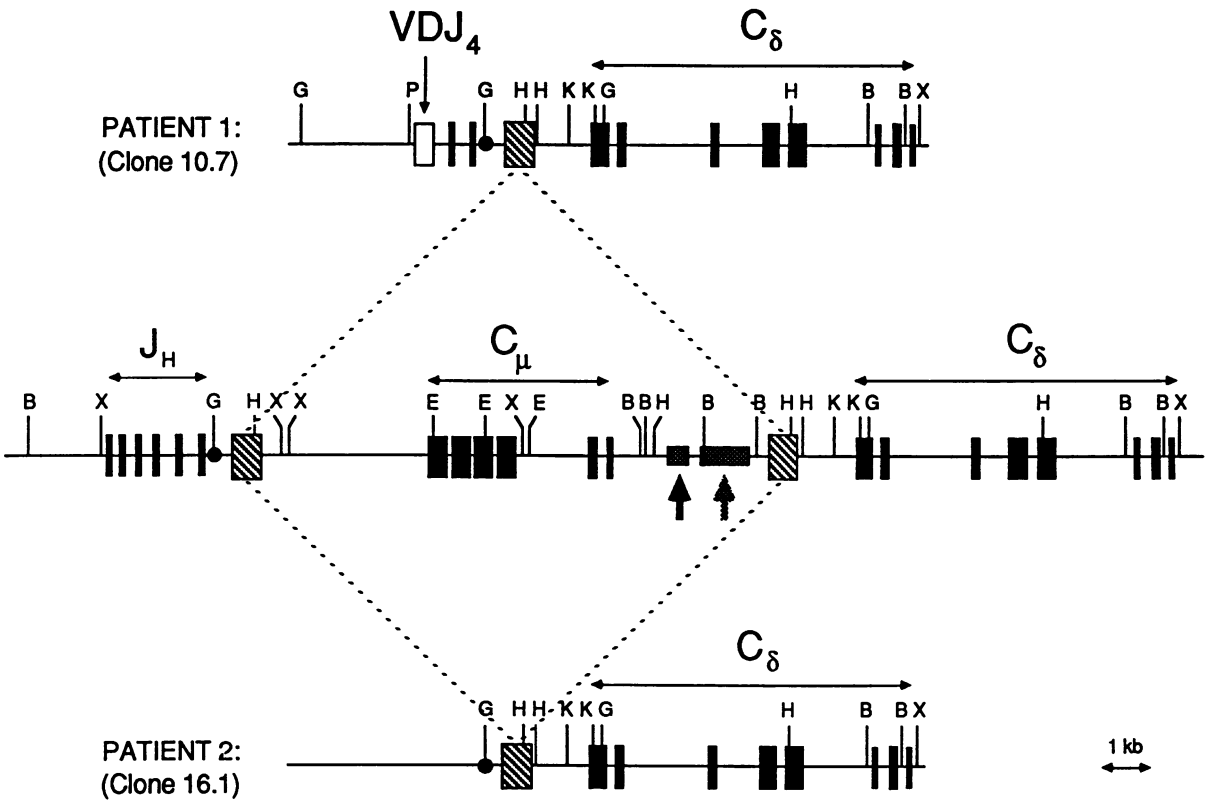


FIG. 1. DNA rearrangements at the immunoglobulin heavy chain locus in two IgD myeloma patients. The restriction map and configuration of the germ line J_H-C_μ-C_δ region is presented in the middle of the figure, with the corresponding regions from patients 1 and 2 presented above and below, respectively. The procedure for the isolation of germ line clones for this region is described elsewhere (60, 61). The procedure for isolating the clones from these IgD myeloma patients is presented in Materials and Methods. Abbreviations: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; X, *Xba*I. Symbols: ■, exons; ▧, locations of the 442/443-bp repeat; ▩, locations of sequences of interest in the C_μ-C_δ intron (black arrow, a 35-mer repeated 12 times in tandem [61]; gray arrow, the region containing a high density of C_μ switchlike pentamers [TGGGG]); ●, immunoglobulin heavy chain enhancer; □, the rearranged VDJ_H region cloned from patient 1; ·····, the C_μ gene deletion event via the 442/443-bp repeat.

patient 1 IgD myeloma cells. If this C_μ gene deletion event is responsible for IgD secretion by these primary myeloma cells, it must be demonstrated that the deletion occurs on the expressed heavy chain allele. Unfortunately, clone 16.1 from patient 2 did not extend far enough 5′ to test for a rearranged V_H. However, Southern hybridization experiments using a probe containing V_H251, a cloned human germ line V_H gene (51), demonstrated that V_H-hybridizing sequences did exist at the 5′ end of clone 10.7 (data not shown). Analysis of the DNA sequence from the resulting subclone revealed the presence of an assembled VDJ_H gene upstream of the C_δ gene in clone 10.7. The DNA sequence of this region is presented in Fig. 3.

This assembled VDJ_H gene possessed the features characteristic of a functional V_H gene (39)—a leader sequence with appropriate splicing junctions (29), conserved cysteine residues at amino acid positions 22 and 92 (19), and functionally spliced V_H, D_H, and J_H exons (no in-phase terminators). The V_H gene segment was most similar (80%) at the DNA level to members of the V_HIII gene family. The D_H segment sequence is different from any reported. The J_H segment matches best but not perfectly to the germ line sequence of J_H4 (43). Although definitive proof requires protein data, it is highly probable that the observed 442/443-bp-mediated C_μ deletion occurred on the functional chromosome.

The recombination endpoints are within the 442/443-bp repeat. The 442-bp (upstream) and 443-bp (downstream) repeat regions located on the unexpressed chromosome of patient 1 were cloned by the PCR technique. Primers 1 and 2 were used to amplify a 649-bp fragment containing the upstream repeat, and primers 3 and 4 were used to amplify a 744-bp fragment containing the downstream repeat. The recombined 443-bp region from the expressed allele was amplified with primers 1 and 4 (Fig. 4A). These fragments were excised from agarose gels and cloned into M13, and three or four clones from each of the three regions were sequenced in order to avoid *Taq* polymerase errors. Figure 4B shows the comparison of the DNA sequences of the 442-bp upstream repeat and 443-bp downstream repeat from the unexpressed allele with that of the recombined 443-bp region from the expressed allele. Fifteen mismatches existed between the upstream and recombined repeat regions, while only five mismatches existed between the downstream and recombined repeat regions. Also, the last 271 bp of the recombined repeat agreed perfectly with the downstream repeat. Thus, it appears that the recombined repeat retained most of the downstream 443-bp region, particularly at its 3′ end. We assume that the three positions within the rearranged sequence which differ from both upstream and downstream repeats are polymorphisms.

When sequencing PCR amplification products, the error frequency for *Taq* polymerase, which has been estimated to be 0.25% or 1 in 400 bp (47), must be taken into consider-

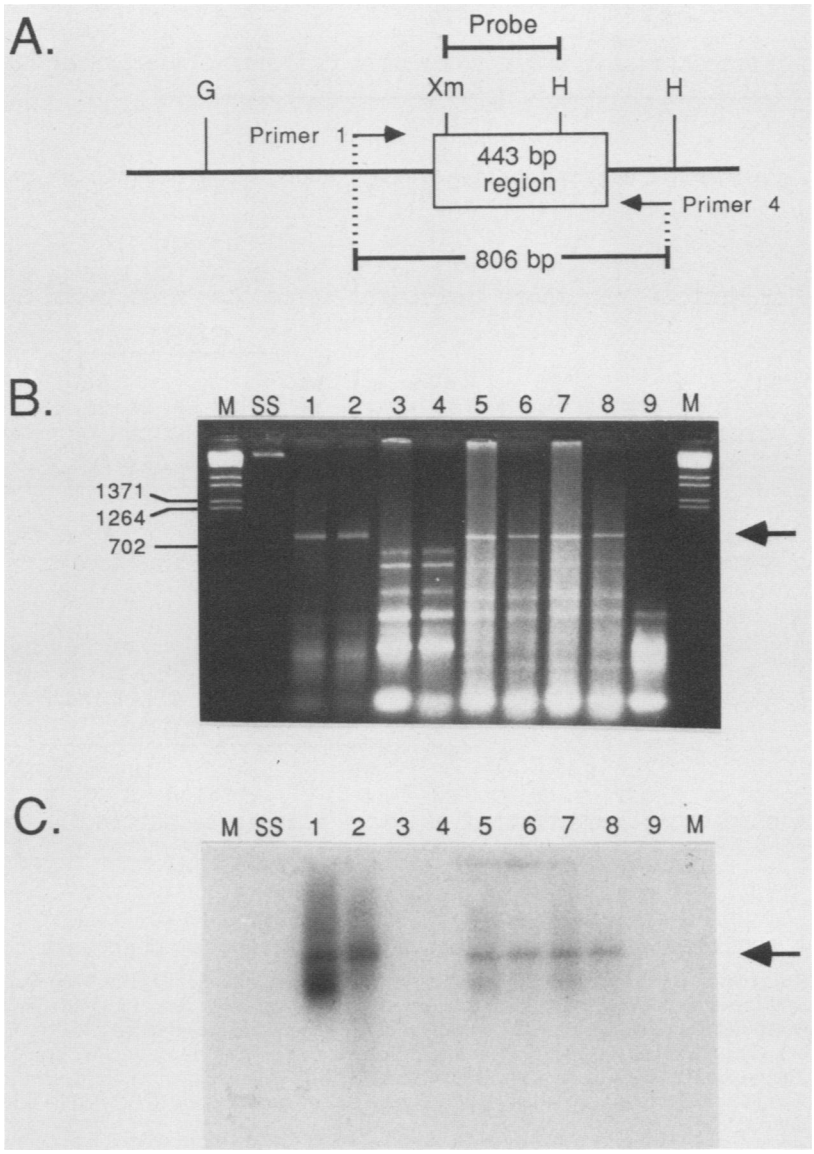


FIG. 2. Analysis of PCR products from two primary IgD-secreting myelomas. (A) PCR strategy for identifying the C_H locus rearrangement in both patients. Abbreviations: G, *Bgl*II; H, *Hin*dIII; Xm, *Xmn*I. The 806-bp fragment amplified by this strategy with primers 1 and 4 is indicated, as is the 285-bp *Xmn*I-*Hin*dIII fragment used as a probe in the following hybridization experiment. (B) Ethidium bromide-stained, 2% agarose gel fractionation of the PCR products from 50 pg of clone 10.7 DNA (lanes 1 and 2), 2 μg of human fibroblast DNA (lanes 3 and 4), 2 μg of patient 1 bone marrow DNA (lanes 5 and 6), 2 μg of patient 2 bone marrow DNA (lanes 7 and 8), and no DNA (lane 9). Lanes M, Marker lanes (*Bst*EII-digested lambda DNA); lane SS, 1 μg of single-stranded M13 DNA. Sizes are indicated on the left in base pairs. The 806-bp fragment amplified in the IgD myeloma samples is indicated by an arrow. The PCR products in lanes 2, 4, 6, and 8 were treated with MBN prior to electrophoresis. (C) The agarose gel from panel B was Southern blotted, hybridized to the ^{32}P-labeled *Xmn*I-*Hin*dIII fragment as a probe, and subjected to autoradiography for 30 min.

ation. This is because any errors occurring in one of the first few rounds of amplification will be copied in all of the remaining rounds and therefore could comprise a large proportion of the resulting PCR product. These PCRs were performed on 1 μg of genomic DNA, which should have consisted of at least 10^5 genomes. Thus, each primer set should have had 10^5 amplification targets in the first round. We feel that by starting with such a large number of target genomes, the resulting PCR products should not contain a significantly high proportion of errors.

Recombination occurs within the 442/443-bp repeats in normal B cells. The finding of homologous recombination in the primary myeloma isolates prompted us to screen normal tissue. Using the PCR strategy of Fig. 2, we analyzed B-cell-enriched and -unenriched fractions from spleen and peripheral blood (Fig. 5). Relatively strong, specific signals were obtained in amplified DNA from enriched PBL (lanes 3 and 7). Products of appropriate sizes and hybridization characteristics were not obtained in unfractionated lymphoid tissues (lanes 1 and 2), nor in human fibroblasts (lane 4) nor in B cells fractionated from mouse splenocytes (lane 6). We conclude that homologous recombination of the 442/443-bp repeat occurs in normal B cells, albeit at relatively low levels. Since there was no recombination in nonlymphoid

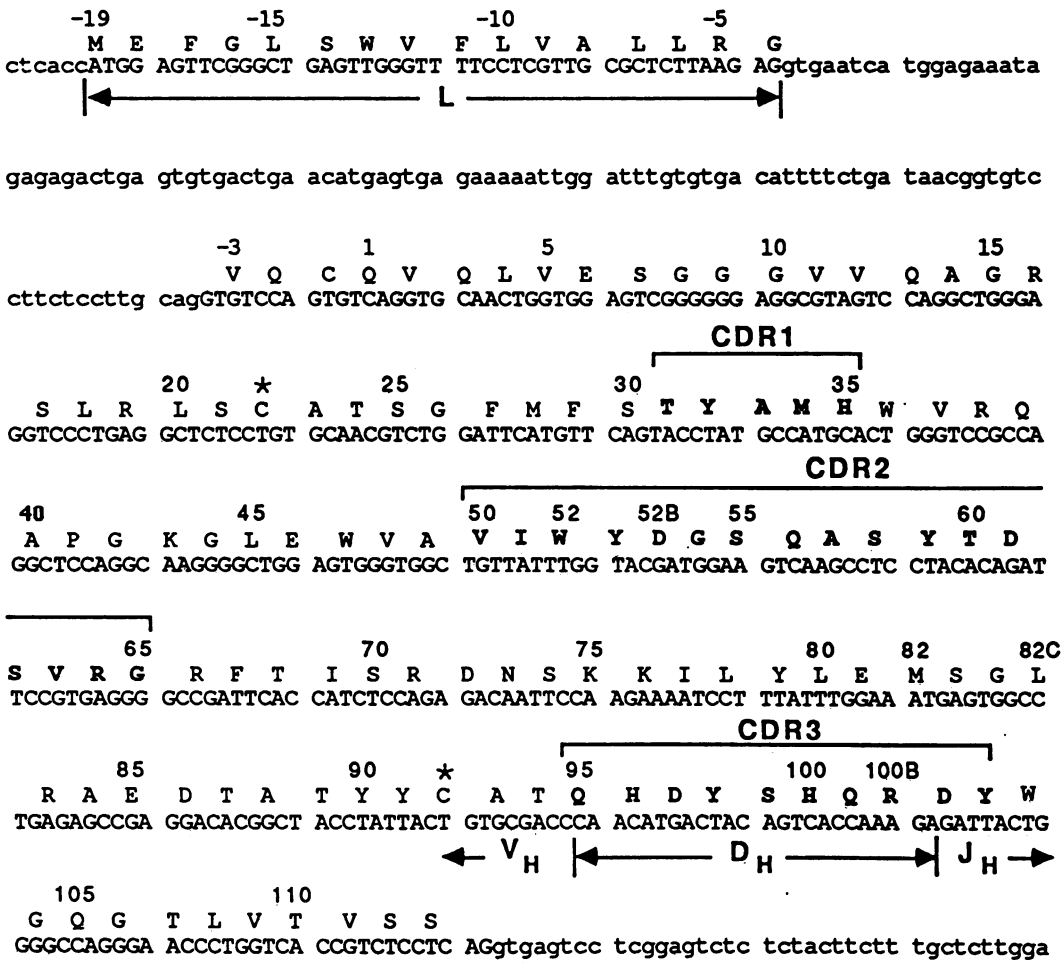
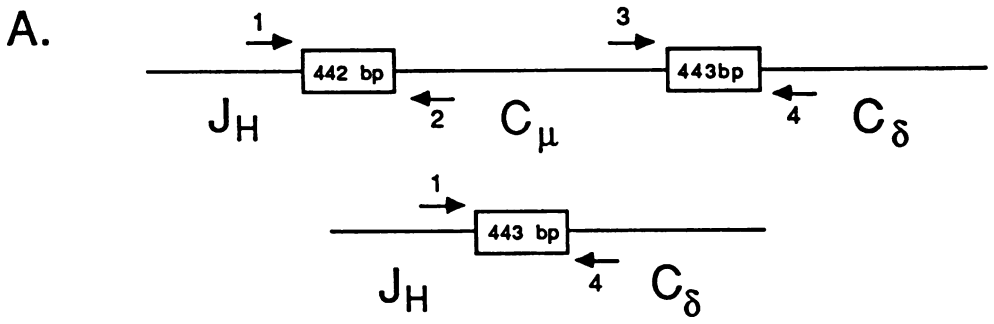


FIG. 3. DNA sequence of the rearranged V_H gene located upstream of the C_δ gene in patient 1. DNA sequences composing the exons are indicated by capital letters, and those composing the introns are indicated by lowercase letters. Predicted amino acid residues are shown above the first base of the corresponding codon. Single-letter abbreviations for amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. Numbering for amino acid residues is according to the method of Kabat et al. (19). Residues labeled with negative numbers represent the leader (L) segment of the V_H region. Amino acid residues in boldface represent the complementary determining regions (CDRs). The conserved cysteine residues at positions 22 and 92 are indicated (*).

tissue and there are no additional copies of the 442/443-bp repeat in the human genome (61), it follows that the primary biologic function of these sequences is to delete C_μ.

DNA recombination 3′ endpoints in two IgD-secreting myeloma cell lines map outside the 442/443-bp and pentamer repeats. In addition to the primary myeloma and normal B-cell isolates, we analyzed the only two well-established IgD-secreting cell lines we could locate. The D64223 line is a hybridoma which resulted from the fusion of bone marrow cells from a patient with IgD myeloma to the murine P3X63Ag8.653 fusion line (J. Kearney, personal communication). Fortunately, only the expressed human C_δ allele was retained in this human-mouse hybridoma (J. Kearney, personal communication), which greatly simplified the analyses. When inserts from C_μ or from several other C_μ-C_δ intron subclones were used as probes on Southern blots of D64223 DNA, no hybridization was seen (data not shown). This suggested that a DNA rearrangement had occurred in this line which deleted the C_μ gene and much of the C_μ-C_δ intervening sequence. Further probing with fragments isolated from pCW35 (Fig. 6a, lanes 1 to 8) and from pCW13 (lanes 9 to 11) placed the recombination between the *Bst*EII site and the *Bgl*II site of $C_{\delta 1}$ (Fig. 6c). Finer mapping with a fragment from pCW101 (Fig. 6a, lanes 12 to 23) reduced the region to ~340 bp between the *Bst*EII and *Xmn*I sites (Fig. 6c) 2 kbp 5′ to the C_δ gene. This recombination site, indicated on Fig. 6c as D6, is considerably downstream of the region of highly concentrated TGGGG pentamers and just 5′ to the 442/443-bp repeat.

Analysis of the DNA from the ODA line (54) was complicated by the presence of the unexpressed H-chain locus and the fact that, unlike D64223, all or most of the human chromosomes present in the ODA line gave rise to background or additional bands or both with the C_μ-C_δ intronic probes. In ODA DNA, various digests probed with pCW35 gave only germ line hybridization (data not shown). However, *Pst*I digestions hybridized to probes progressively 5′ to pCW35 (pCW101, pAS13, and pCW13) gave a rearranged band of 8.5 kbp (Fig. 6b, lanes 1 to 12). Indeed, the *Pst*I rearrangement was detected by a fragment from the 3′ end of pCW2, indicating that the recombination occurred far upstream of the site used in D64223. Additional digestions (Fig.



B.

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                    10        20        30        40        50        60
                    |         |         |         |         |         |
UPSTREAM            ----------------------G-----------------------------------
PATIENT #1          ATCATTATCCTGAATTATTTCAGTTAAACATGTTAGTTGGTGGCATAAGAGAAAACTCAATCAG
DOWNSTREAM          -------C------------------G-------------------------------G----

                    70        80        90        100       110       120
                    |         |         |         |         |         |
UPSTREAM            --A-------------------------------------------C-------C---
PATIENT #1          ATGGTGCTGAAGACAGGACTGTGGAGACACCTTAGAAGGACAGATTCTGTTCAGAATCACTGAT
DOWNSTREAM          --A-----------------------------------------------------------

                    130       140       150       160       170       180       190
                    |         |         |         |         |         |         |
UPSTREAM            ------------------------C-----------------G---------------------
PATIENT #1          GCGGCGTCAGCTGGACTGGCCTAGTGGAGGCTCTGGGAGGGTGACTGCCAGGCCCGGCCTGGGC
DOWNSTREAM          -------------------------------------------G-------------------

                    200       210       220       230       240       250
                    |         |         |         |         |         |
UPSTREAM            ---------------------A-----------------------T-----------------
PATIENT #1          TTTGGGTCTCCCCGGACTACCCGGAGCTGGGATGCGTGGCTTCTGCCGCCGGGGCCGACTGGCTG
DOWNSTREAM          -----------------------------------------------------------------

                    260       270       280       290       300       310       320
                    |         |         |         |         |         |         |
UPSTREAM            ------------------------------------------------------ ------C
PATIENT #1          CTCAGGCCCCAGCCCTTGTTAATGGACTTGGAGGAATGATTCCATGCCAAAGCTTTTGCAAGGT
DOWNSTREAM          -----------------------------------------------------------------

                    330       340       350       360       370       380
                    |         |         |         |         |         |
UPSTREAM            ----------G------------------------------------------TG------
PATIENT #1          TCGCAGTGACCAGGCACCCGACATGGTAAGAGACAGGCAGCCGCCGCTGCTGCATTCACTTCTC
DOWNSTREAM          -----------------------------------------------------------------

                    390       400       410       420       430       440
                    |         |         |         |         |         |
UPSTREAM            ----------------C-------------------G---C-----------------
PATIENT #1          TTAAAACTTTGTATTTGATGTCTTATTTCCACTAGAAGGCGAATTGGTCTTAATTGCTT
DOWNSTREAM          -----------------------------------------------------------
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FIG. 4. PCR amplification and sequencing of the germ line 442/443-bp repeat regions from patient 1. (A) The top line shows the unexpressed chromosome of patient 1 and the locations of the primers used to amplify the 442- and 443-bp repeat regions from this allele. The bottom line shows the expressed chromosome and the locations of the primers used to amplify the recombined 443-bp repeat region. (B) The middle line shows the DNA sequence of the recombined 443-bp sequence from the expressed allele. The 442-bp upstream and 443-bp downstream repeat sequences are shown above and below this line, respectively. Matching base pairs are indicated by dashes.

6b, lanes 13 to 17; also data not shown) have narrowed the deletion endpoint (indicated by ODA and brackets in Fig. 6c) between the *Rsa*I and *Sph*I sites, just downstream of the μM poly(A) site. As with D64223, neither the 442/443-bp repeat nor the TGGGG pentamers appeared to be involved in the recombination.

DISCUSSION

In humans, IgD myeloma composes only 1 to 2% of all myeloma cases (17). The finding of two patients diagnosed as having IgD-secreting myeloma has provided a rare opportunity to study the DNA rearrangements leading to IgD

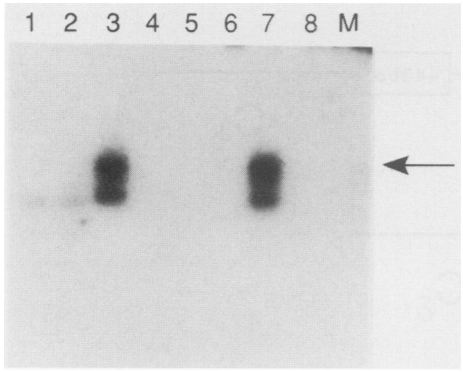


FIG. 5. The 442/443-bp repeat recombines in normal B cells. Approximately 2 μg of DNA from B and non-B sources was amplified, fractionated, and probed as described in the legend of Fig. 2 for fusions of the 442- and 443-bp sequences. DNA was prepared from unpurified human PBL (lane 1), unpurified human splenocytes (lane 2), separate human PBL samples enriched for B cells (lanes 3 and 7), mouse splenocytes (lane 4), and human fibroblasts (lanes 5 and 6). Lane 8 contained no DNA, and lane M contained markers. The arrow denotes the predicted 806-bp fragment which is resistant (data not shown) to MBN.

secretion by these cells at a primary stage of disease. Our studies of these myeloma cells at the DNA level demonstrated that the C_μ gene has been deleted via a 442/443-bp direct repeat. The recombination event leading to the C_μ gene deletion occurred between the two copies of this repeat present on either side of this gene. One complete 443-bp copy of the repeat was retained. Because the unrearranged repeat copies were not completely identical (96% similar), we were able to determine approximately the contribution of each unrearranged repeat region to the recombined repeat region. Analysis of our sequencing data demonstrated that the recombined 443-bp region in patient 1 myeloma cells contained portions of both unrearranged regions but was composed mainly of the downstream 443-bp region.

The immunoglobulin class switch has been shown to occur at or near the switch (S) sites of the heavy chain genes involved in the recombination event (52). These S regions are composed of tandem arrays of direct repeated sequences. The recombination event leading to IgD secretion by the myeloma cells resembled the classic C_H class switch in that the C_H gene to be expressed (C_δ) was brought adjacent to the VDJ_H assembly. However, the composition of the C_δ repeat sequence is quite different from that of the C_H switch sites. The C_δ switchlike regions consist of only one long direct repeat that was not repeated in tandem. The 442/443-bp repeat is highly similar (96%), whereas S_μ shared only about 65% similarity with other S regions. As a result, the homologous recombination event resulting in the C_μ deletion was very specific, while class switches to the other C_H genes have been shown to occur at several positions within the S regions and even just outside of them. Previous Southern blotting experiments on human genomic DNA have demonstrated that this repeat is unique to the J_H-C_μ-C_δ locus (61). Also, DNA sequence comparisons of the 442/443-bp repeat with the repeats of the other C_H switch sites or with any other data base entries gave no significant similarities.

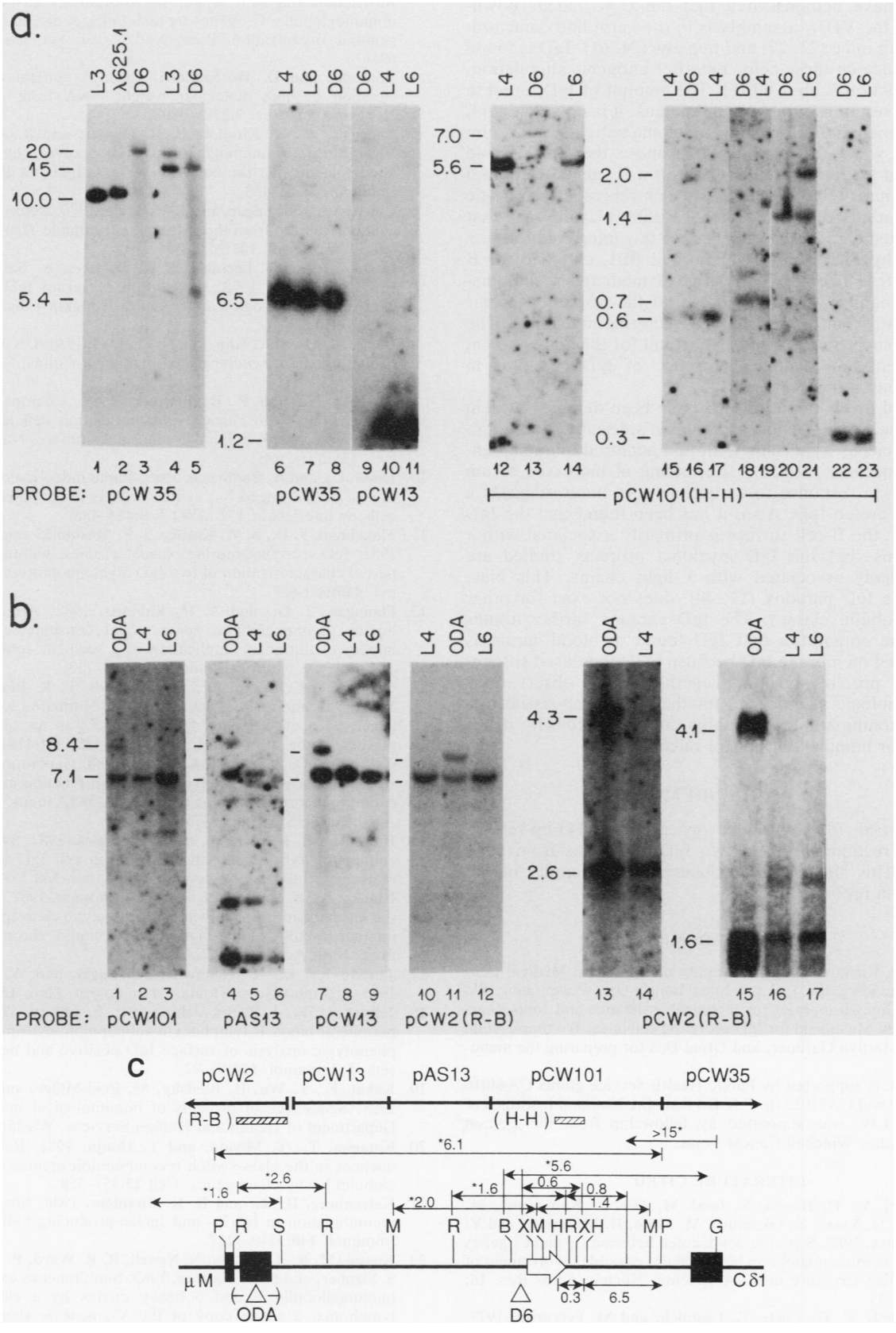
The recombination of S_μ with downstream S regions in classical switches resulted in the deletion of only part of the S_μ region. It has been shown that the remaining portion of the S_μ region is able to mediate progressive class switches to further downstream C_H genes (14, 15). The switch from C_μ to C_δ in these myeloma cells results in the complete deletion of the S_μ region. Thus, it would be expected that the C_δ switchlike event occurring in these IgD myeloma cells would prevent any further class switching.

Given the specificity of homologous recombination, it was unexpected that C_μ deletion apparently can still occur through illegitimate recombination. Analysis of two established IgD-secreting cell lines showed that the 3′ deletion endpoint was not within the C_μ-C_δ intron component of the 442/443-bp repeat. Clearly for ODA, homologous recombination was not implicated in the C_μ deletion which occurred far upstream near the Cμ membrane (μm) exons. However, the D64223 3′ endpoint was just 5′ (within 50 to 100 bp) of the repeat. Endpoints of classical switch recombinations involving S_μ often map 5′ of the tandem pentamer repeat region (e.g., see Fig. 9 of reference 21 for a review). This has been interpreted to represent secondary recombination endpoints, following progressive deletion around the primary recombination sites (within the repeat unit) during propagation of the immortalized cells. Another explanation is that the S_μ-associated repeats were involved in the initial pairing between switch regions, but the actual site of cleavage and religation can occur upstream. Either interpretation could apply to the observed deletion in D64223.

Homologous recombination of 442/443-bp repeats in fibroblasts was not observed, suggesting some type of cell type regulation. The VDJ-C_μ-C_δ locus is transcriptionally active only in B cells. There are several precedents for the correlation between transcription and recombination in yeasts (59) and in higher eucaryotes (10, 62). An altered chromatin structure may result from transcription, increasing the accessibility of the repeats to generalized recombinational machinery. Alternatively, the altered structure may be more sensitive to an endonucleolytic cleavage that is resolved by recombination.

The main function attributed to IgD in the vertebrate immune system is as an antigen receptor on mature, virgin B

FIG. 6. DNA rearrangements in two IgD-secreting myeloma cell lines map within the C_μ-C_δ intron but outside of the 442/443-bp repeat. (a) Comparative Southern hybridization of D64223 (D6) and liver (L) DNA. λ625.1 is a phage clone that contains the entire C_δ gene and C_μ-C_δ intron (61). Digestions with *Bam*HI (lanes 1 to 3), *Bgl*II (lanes 4 and 5), *Hin*dIII (lanes 6 and 7), and *Bst*EII (lanes 9 to 11) were used to approximately position the rearrangement. Locations of probes are indicated in panel c. H-H, A 1-kbp *Hin*dIII-*Hin*dIII fragment from pCW101. Digestions were with *Bgl*II (lanes 12 to 14), *Xmn*I (lanes 15 to 17), *Rsa*I (lanes 18 and 19), *Msp*I (lanes 20 and 21), and *Hin*dIII (lanes 22 to 23). (b) Comparative Southern hybridization of ODA and liver (L) DNA (L3, L4, and L6 were from different individuals and showed no apparent differences). Lanes 1 to 12, *Pst*I digestions probed with sequences located progressively 3′ to C_δ. pCW2(R-B), The −300-bp *Rsa*I-*Bgl*I fragment derived from pCW2 (c). A more precise location of the deletion is revealed by digestion with enzymes within pCW101: *Rsa*I (lanes 13 and 14) and *Sph*I (lanes 15 to 17). (c) Positions of probes and summary of rearrangement analysis. The line at the top represents inserts from plasmids subcloned from λ625.1 (61); subfragment probes are indicated below (▨). Lengths of restriction fragments that correspond to the data in panels a and b are shown above and below the scaled map. Boxes are exons, and the large rightward arrow is the downstream copy of the 442/443-bp repeat. Asterisks denote rearranged fragment sizes. Enzymes are abbreviated as in Fig. 1, except for the following: X, *Xmn*I; R, *Rsa*I; S, *Sph*I; B, *Bst*EII; M, *Msp*I. The positions of the D64223 and ODA deletion endpoints are indicated (△).


a.
L3
λ625.1
D6
L3
D6
L4
L6
D6
D6
L4
L6
L4
D6
L6
L4
D6
L4
D6
L4
D6
L6
D6
L6
20
15
10.0
5.4
6.5
1.2
7.0
5.6
2.0
1.4
0.7
0.6
0.3
1 2 3 4 5
6 7 8 9 10 11
12 13 14
15 16 17 18 19 20 21 22 23
PROBE: pCW35
pCW35 pCW13
pCW101(H-H)
b.
ODA
L4
L6
8.4
7.1
4.3
2.6
4.1
1.6
1 2 3
4 5 6
7 8 9
10 11 12
13 14
15 16 17
PROBE: pCW101
pAS13
pCW13
pCW2(R-B)
pCW2(R-B)
c
pCW2
pCW13
pAS13
pCW101
pCW35
(R-B)
(H-H)
*6.1
>15*
*5.6
*2.6
*1.6
0.6
0.8
1.4
*2.0
P R S R M R B X M H R X H M P G
μM
ODA
D6
0.3
6.5
Cδ1

cells. Studies of B-cell lymphomas expressing surface IgM and IgD have demonstrated that the C_μ-C_δ locus downstream of the VDJ_H assembly is in the germ line configuration in both mice (22, 27) and humans (24, 61). IgD is found in abundance on these cells, but after antigenic stimulation, IgD production is shut off (5). The amount of IgD found in the blood serum is quite low; in humans, it is only 60 μg/ml, on the average (46). In our IgD myeloma patients, the serum IgD level was ~20 mg/ml. We propose that this 33-fold increase in serum IgD is the result of a C_μ gene deletion in the bone marrow myeloma cells which represent neoplastic transformations of normal IgD-secreting plasma cells that have deleted C_μ. Direct evidence for this interpretation was provided by PCR analysis of normal PBL enriched for B lymphocytes. The 442/443-bp repeat mediated recombination in a manner indistinguishable, at the resolution of our experiment, from that of the IgD-secreting myelomas. The finding of such B cells also can account for the higher serum IgD content and higher occurrence of IgD myeloma in humans than in mice.

Elevated levels of serum IgD have been demonstrated in certain disease states such as Hodgkin's disease (7), autoimmune deficiency syndrome (32), and acquired immune deficiency syndrome (32, 38). The finding of increased serum IgD levels in response to certain disease states suggests a role for secreted IgD. Also, it has been found that the IgD present on the B-cell surface is primarily associated with κ light chains, but the IgD myeloma proteins studied are predominately associated with λ light chains. This bias, termed the IgD paradox (17, 40), does not exist for other immunoglobulin classes. The IgD paradox further argues against the contention that IgD found in blood serum is merely shed membrane IgD because of its increased susceptibility to proteolysis (16). Together, these observations provide biologic significance for the 442/443-bp-repeat-mediated recombination. It remains unclear as to why it has evolved for humans but not for mice.

ADDENDUM

Yasui et al. (63) have also observed 442/443-bp-repeat-mediated recombination in two IgD myelomas from bone marrow. This significantly enhances the generality of the observation reported here.

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