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Nonhomologous Recombination at Sites within the Mouse J_H-C_δ Locus Accompanies Cμ Deletion and Switch to Immunoglobulin D Secretion

JAMES D. OWENS, JR.,[1] FRED D. FINKELMAN,[2] JOHN D. MOUNTZ,[3] AND J. FREDERIC MUSHINSKI[1]*

Laboratory of Genetics, National Cancer Institute, Bethesda, Maryland 20892[1]; Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814[2]; and Department of Medicine, University of Alabama, Birmingham, Alabama 35294[3]

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Plasma cells secrete immunoglobulins other than immunoglobulin M (IgM) after a deletion and recombination in which a portion of the immunoglobulin heavy-chain locus (IgH), from the 5′-flanking region of the μ constant-region gene (C_μ) to the 5′-flanking region of the secreted heavy-chain constant-region gene (C_H), is deleted. The recombination step is believed to be targeted via switch regions, stretches of repetitive DNA which lie in the 5′ flank of all C_H genes except δ. Although serum levels of IgD are very low, particularly in the mouse, IgD-secreting plasmacytomas of BALB/c and C57BL/6 mice are known. In an earlier study of two BALB/c IgD-secreting hybridomas, we reported that both had deleted the C_μ gene, and we concluded that this deletion was common in the normal generation of IgD-secreting cells. To learn how such switch recombinations occur in the absence of a switch region upstream of the $C_{\delta 1}$ exon, we isolated seven more BALB/c and two C57BL/6 IgD-secreting hybridomas. We determined the DNA sequences of the switch recombination junctions in eight of these hybridomas as well as that of the C57BL/6 hybridoma B1-8.δ1 and of the BALB/c, IgD-secreting plasmacytoma TEPC 1033. All of the lines had deleted the C_μ gene, and three had deleted the $C_{\delta 1}$ exon in the switch recombination event. The delta switch recombination junction sequences were similar to those of published productive switch recombinations occurring 5′ to other heavy-chain genes, suggesting that nonhomologous, illegitimate recombination is utilized whenever the heavy-chain switch region is involved in recombination.

Synthesis of immunoglobulin (Ig) molecules by members of the B-cell lineage involves a series of somatic gene rearrangements and deletions which occur in an ordered fashion during B-cell development. Two site-specific recombination events fuse heavy-chain variable-region, diversity, and joining gene elements (V_H, D, and J_H, respectively) to make a functional μ heavy-chain gene during differentiation of hematopoietic stem cells into pre-B cells. A similar site-specific recombination produces a functional κ or λ light-chain gene as pre-B cells differentiate into immature B cells that express surface IgM. When stimulated by antigen or mitogen, B cells mature into plasma cells that initially secrete IgM but later secrete Igs containing the same V_H-D-J_H associated with other heavy-chain classes.

The switch from μ, the 5′-most heavy-chain constant-region gene, to other heavy-chain classes is accomplished by another somatic rearrangement of the heavy-chain locus, the switch recombination. In switch recombination, a 5′ breakpoint upstream of the C_μ gene is joined to a 3′ breakpoint upstream of another C_H gene, and the intervening DNA is deleted. The switch recombination has been linked to switch regions: 2 to 5 kb of tandem repeats in which the tetramers AGCT and TGGG occur frequently (46). Switch regions are found in the 5′-flanking area of each C_H gene except C_δ. Curiously, most switch recombinations do not have their 5′ breakpoints in the switch μ region (S_μ), though the 3′ breakpoints usually are in the switch region of the expressed C_H gene (25).

Since IgD is rare in human serum and very rare in mouse serum, it was believed that normal production of secreted IgD might not require switch recombination but might occur by alternative splicing of transcripts spanning the V_H-D-J_H and C_μ-C_δ locus, the same mechanism utilized for membrane IgD (reviewed in reference 6). Although human B cells with intact C_μ genes can synthesize small but significant amounts of mRNA for secreted δ chain (30), this is not the case in mice (15). It is likely that secretion of large amounts of IgD requires some sort of recombination, since three cultured human cell lines and three human myelomas (72, 75), three mouse cell lines (26, 54), and two murine plasmacytomas (17) that secrete IgD have deleted the C_μ gene from the active chromosome. In human beings, switch recombination to δ can occur between two nearly perfect 443-bp repeats upstream and downstream of the C_μ gene (70, 75), but there is no such repeat in the much smaller C_μ-C_δ intron of the mouse (48). We wished to find out whether switch recombinations to δ occur in murine splenocytes and, if so, how the switch recombination sites compared with those of switch recombinations to other classes of heavy-chain genes.

Injection of mice with goat anti-mouse δ antibody induced a 100-fold increase in the number of IgD-secreting splenocytes 7 to 8 days postinjection (44). Fusion of spleen cells from these mice with a non-Ig-secreting plasmacytoma allowed selection of IgD-secreting hybridomas. We have previously analyzed two such hybridomas and demonstrated that one, KWD2, produced normal-sized δ-chain mRNAs and proteins, while the other, KWD1, produced shortened δ-chain mRNAs and proteins (43). Both hybridomas had deleted the C_μ gene. The shortened δ chain of KWD1 had a functional variable region, but the constant region lacked the $C_{\delta 1}$ exon. The deletion of the $C_{\delta 1}$ domain in KWD1 was not due to in vitro manipulation, since at 8 days after injection of

* Corresponding author.

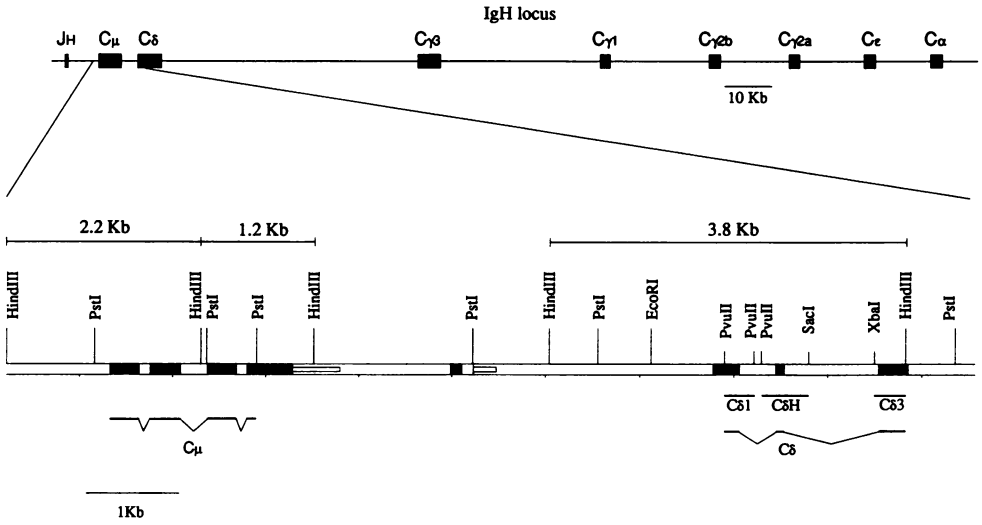

FIG. 1. Murine heavy-chain locus with the μ-δ region expanded to show the probes used in this work. In the expanded region, black boxes indicate coding sequences, thin white boxes represent transcribed but untranslated areas, and thick white boxes represent introns. This restriction map is complete for the *Eco*RI, *Hin*dIII, and *Pst*I sites only; the *Xba*I, *Sac*I, and *Pvu*II sites shown are those used to generate probes they delimit. Sizes of the germ line *Hin*dIII fragments detectable with these probes in Fig. 3 and Table 3 are indicated.

goat anti-mouse δ antibody approximately 25% of secretory δ-chain mRNA in the spleen lacked the $C_{\delta 1}$ exon.

In this paper, we report the DNA sequence of the switch recombination sites of these two hybridomas, of seven more hybridomas, and of an IgD-secreting plasmacytoma. All have undergone switch recombinations in which the C_μ gene was lost, and in three the $C_{\delta 1}$ exon has been lost as well. These switch recombination sites are compared with other known switch recombination sites. We conclude that switch recombination is necessary for secretion of murine IgD, that this switch recombination is indistinguishable from that to other classes, and that switch recombination utilizes a form of nonhomologous, illegitimate recombination which is involved in random integration of foreign DNA into the genome. This permits a hypothesis unifying normal switch recombination and chromosomal translocations involved in transformed lymphocytes.

MATERIALS AND METHODS

DNA probes. The probes used are shown in Fig. 1. The C_δ probe was a 563-bp *Pvu*II-*Hin*dIII fragment from the cDNA clone pδ54J (65). Genomic probes for individual C_δ exons were as follows: $C_{\delta 1}$ probe, the 322-bp *Pvu*II fragment; $C_{\delta H}$ probe, the 520-bp *Pvu*II-*Sac*I fragment; and $C_{\delta 3}$ probe, the 344-bp *Xba*I-*Hin*dIII fragment. The C_μ probe was the 1,190-bp *Pst*I fragment from the cDNA clone pMKμ1 (27).

Generation of IgD-secreting hybridomas. The generation, screening, and cloning of the IgD-secreting hybridomas KWD1 and KWD2 were described previously (43). Additional hybridomas were produced by the same procedure except that the spleen cells were fused to Sp2/0-Ag14 (Sp2/0) cells (57) instead of the P3X63Ag8653.6 (P3X) cells used to generate KWD1 and KWD2. Spleen cells were obtained from anti-IgD-treated BALB/c mice for the KWD series of hybridomas and from C57BL/6 mice for hybridomas 2C8 and 4C8.

RNA preparation and blotting. RNA was prepared by a variation of the guanidinium thiocyanate method (66). Approximately 2×10^8 cultured cells were gently pelleted and resuspended in 7.7 ml of 4 M guanidinium thiocyanate–0.1 M Tris-HCl (pH 7.5)–0.1 M β-mercaptoethanol–0.5% sodium lauryl sarcosinate. The cells were broken open by shearing with a Polytron homogenizer (Brinkmann) at the highest speed for 45 s. Debris was removed by a brief, gentle centrifugation, and the supernatant was layered over 4 ml of 5.7 M CsCl–4 mM EDTA (pH 8) and spun for 16 h at 80,000 × *g* and 22°C. The supernatant was carefully aspirated and discarded. The RNA pellet was dissolved in 400 μl of 10 mM sodium acetate (pH 5)–5 mM EDTA–5% phenol and then was extracted with 0.5 ml of phenol (saturated with 50 mM sodium acetate [pH 5]) by rocking for 1 h. Then 0.5 ml of $CHCl_3$ was added, and the contents of the tube were mixed by vortexing. After a 5-min centrifugation, the aqueous phase was reextracted with an equal volume of $CHCl_3$; then the RNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. Enrichment of poly(A)$^+$ RNA was accomplished by chromatography on a column of oligo(dT)-cellulose (2).

RNA was denatured in formaldehyde-formamide and analyzed by electrophoresis on a 1% agarose–formaldehyde gel (18, 33). Five micrograms of poly(A)-enriched or 10 μg of total RNA was applied per well. The RNAs were blotted onto nitrocellulose or nylon membranes, which were then hybridized with DNA probes labeled by nick translation (49) or random oligonucleotide priming and elongation in the presence of [^{32}P]dCTP (14).

DNA preparation and blotting. Hybridoma DNA was prepared by a variation of the method of Blin and Stafford (8). Approximately 10^8 cultured cells were pelleted and resuspended in 50 ml of 0.1 M NaCl–50 mM EDTA (pH 8)–50 mM Tris-HCl (pH 7.5) before addition of 1.2 ml of 20% sodium dodecyl sulfate and 7 mg of proteinase K (Boehringer Mannheim). Digestion proceeded overnight at 55°C without shaking before extraction with an equal volume of phenol-$CHCl_3$-isoamyl alcohol (1:1:0.1, vol/vol/vol). High-molecular-weight DNA was spooled onto a pipet from the aqueous phase which had been overlaid with ethanol. The DNA was dissolved in TE (0.01 M Tris-HCl [pH 7.5], 1 mM EDTA), digested with RNase A and then with proteinase K,

TABLE 1. Oligonucleotides used for PCR and sequencing

Used for:	Oligonucleotide no.	Nucleotides[a]	Sequence
KWD1	525	5139–5162	CAGCCTCGGT GGCTTTGAAG GAAC
	434	18406′–18381′	CGATGGTGAG AGATCAGAGT ATTCCG
KWD2	524	4995–5018	CTGGCCCTGC TTATTGTTGA ATGG
	522	16063′–16041′	GGCACACCCA CATACCACAA AGC
KWD4	521	17224′–17200′	CAGAGTCACC TCTATACCCT ATGTC
KWD5	564	6033–6056	GGAACAAGGT TGAGAGCCCT AGTA
	747	15598′–15575′	CACTGTTCCA TTTCCAGTTC CTAG
KWD6	431	5422–5446	GCTCATGGTA TTTTGAGGAA ATCTT
	432	18900′–18877′	CCTTTAACGA GATTCTTATG CGCC
KWD8	425	16854′–16832′	GGGATAAGAC CCAAATTACT AGC
KWD9	563	5268–5291	CTGCTTAAGA GGGACTGAGT CTTC
	425	16854′–16832′	GGGATAAGAC CCAAATTACT AGC
2C8	427	2980–3004	GCTTGAAGTC TGAGGCAGAA TCTTG
	434	18406′–18381′	CGATGGTGAG AGATCAGAGT ATTCCG
B1-8.δ1	748	10948–10971	CAGCCAGTGT CTCAGAGGGA AGCC
	749	17015′–16991′	GTGGAACCTA GTGGAGATGA ACTTG
TEPC 1033	524	4995–5018	CTGGCCCTGC TTATTGTTGA ATGG
	436	17934′–17908′	CAGAGGGGAA GACATGTTCA ACTATAC

[a] Nucleotide numbering is that of the 28.2-kb sequence, SGENOME5, obtained from Philip Tucker. This numbering was used because not all of the oligonucleotides are in GenBank entries. SGENOME5 bases 1013 to ~6570 are found in locus MUSIGCD07 (accession number J00440), bases 11223 to 13695 are in locus MUSIGCD10 (accession number J00443), bases 13124 to 19923 are in locus MUSIGCDB (accession number K02138), and bases 24130 to 28224 are in locus MUSIGCD17 (accession number J00450). Primed bases are from the noncoding strand.

extracted with phenol-$CHCl_3$-isoamyl alcohol as described above, spooled out again, and dissolved in 1 ml of TE.

Bacteriophage λ DNA was prepared by a standard method (38).

Plasmid DNA was prepared by a variation of the alkaline lysis technique (5, 28, 37) except that the neutralizing solution was 3 M potassium acetate–1.8 M formic acid (4). The isopropanol precipitate was purified either on a CsCl-ethidium bromide equilibrium density gradient or by pelleting through 3 M NaCl at 300,000 × *g* for 90 min (34) in an SW55Ti rotor.

Restriction endonuclease digestions of 15 μg of DNA were separated on agarose gels, transferred to nitrocellulose membranes, and hybridized to radiolabeled DNA probes (59).

Cloning of δ-hybridizing *Hin*dIII fragments. Sixty micrograms of genomic DNA from each hybridoma was digested with 300 U of *Hin*dIII (New England Biolabs) and fractionated by electrophoresis on 0.8% GTG Seakem agarose gel (FMC) in 0.16 M Tris acetate (pH 7.6)–16 mM EDTA. Fragments approximating the size of the rearranged C_δ-hybridizing fragment for each hybridoma were cut from the gel and purified by a variation of the method of Vogelstein and Gillespie (67), using the Geneclean glassmilk kit (Bio 101).

The *Hin*dIII fragments were ligated to Charon 27 DNA which had been circularized at the cohesive ends and were then linearized with *Hin*dIII and alkaline phosphatase treated as described previously (35). The constructs were packaged in Gigapack-Gold extracts (Stratagene) according to the supplier's directions and transfected into LE392 cells. Plaques were screened until plaque pure with the C_δ probe and then picked and rescreened until all plaques hybridized to the C_δ probe. At this stage, the plaques were considered pure. The *Hin*dIII inserts were subcloned into the *Hin*dIII site of pGEM3Z by standard methods.

PCRs. Not all the switch recombination junctions were successfully cloned in Charon 27. Polymerase chain reactions (PCRs) (55) were used to complete the sequencing of the switch junctions. Sequences of oligonucleotide primers (Table 1) were chosen from a 28.2-kb sequence of the portion of mouse chromosome 12 covering the region from upstream of D_{Q52} to downstream of the $\delta_{membrane}$ exons, determined, compiled, and generously supplied to us by Philip Tucker. Two strategies were used to generate PCR fragments that included the switch recombination junctions. First, fragments were generated that could be made only if the DNA had deleted a substantial region including the C_μ gene. Second, in hybridomas that had only a rearranged copy of the J_H-C_μ-C_δ region, deletions were mapped by determining which PCR primer pairs failed to generate a germ line fragment owing to the deletion of one or both of their templates during the recombination.

PCRs were run with reagents from AmpliTaq kits (Perkin Elmer-Cetus) and oligodeoxynucleotide primers in a total reaction volume of 100 μl at 0.2 mM each deoxynucleotide 5′-triphosphate and 1.0 μM each oligonucleotide primer with 1 μg of genomic DNA template. Denaturation at 94°C for 3 min preceded 30 cycles of 68°C for 30 s, 72°C for 1 min, and 94°C for 15 s in an ERICOMP, Inc., Programmable Cyclic Reactor. The final cycle of each PCR was at 65°C for 30 s and 72°C for 10 min.

Asymmetric PCRs (41) were used to produce single-stranded fragments for sequencing and were carried out as described above, with the following modifications. Deoxynucleotide triphosphate concentrations were reduced to 0.1 mM. The nonlimiting primer concentration was 0.5 μM, and the limiting primer concentration was 0.01 μM, for a ratio of 50:1. The templates were PCR fragments produced from genomic DNA as described above. Sometimes it was possible to use the product of an asymmetric amplification for sequencing reactions directly after ethanol precipitation as described below, without a gel purification, but more consistent results came from fragments purified by phenol extraction from low-melting-point agarose gels (69).

The products of each 100-μl PCR reaction were precipitated by addition of 33 μl of 10 M ammonium acetate and 160 μl of ethanol, rinsed with 1 ml of 70% ethanol, and separated by electrophoresis on low-melting-point agarose gels in 40 mM Tris acetate (pH 7.8)–2 mM EDTA–0.5 μg of ethidium bromide per ml. Bands were visualized by UV

light, excised, melted, and purified by phenol extraction (69). Fragments were dissolved in 10 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA.

We chose not to clone PCR fragments because of the error-prone reputation of *Taq* DNA polymerase (64). Thus, we sequenced the PCR fragments amplified from 10^5 copies of genomic sequence (1 μg of genomic DNA) to minimize the possibility that mistakes in early cycles would contribute ambiguities or mistakes in the sequencing reactions.

Sequencing. The Sequenase kit from United States Biochemical Corp. was used to sequence both plasmids and PCR-generated fragments. The pGEM3Z subclones were sequenced by the NaOH denaturation method (9).

Double-stranded PCR fragments were sequenced as recommended by Bachmann et al. (3). Briefly, 0.5% Nonidet P-40 was present throughout the Sequenase protocol. Gel-purified, PCR-generated DNA was denatured at 95°C for 3 min in the presence of an oligonucleotide primer before rapid cooling in dry ice. As the annealing mixture thawed, the other components of the extension reaction were added, and this mixture was immediately divided four ways for the termination reactions at 37°C for 5 min.

Asymmetrically amplified PCR fragments were sequenced by the method suggested in the Sequenase kit for single-stranded DNA. Autoradiograms of sequencing gels of double-stranded PCR fragments required 2 to 4 weeks of exposure to produce a readable sequencing ladder, but single-stranded PCR fragments required only 16 to 72 h of exposure.

Primers. Oligonucleotides (Table 1) were synthesized for us on an Applied Biosystems model 381A DNA synthesizer. The M13 universal primer was that supplied by United States Biochemical in the Sequenase kit. The M13 reverse sequencing primer was purchased from New England Biolabs (catalog no. 1201).

Enzymes. Restriction endonucleases were purchased from Bethesda Research Laboratories, Boehringer Mannheim Biochemicals, and New England Biolabs and were used in buffers supplied with the enzymes. Other enzymes used were purchased from the same suppliers and were used in buffers recommended by the suppliers.

Nucleotide sequence accession numbers. The novel sequences of the switch recombination sites were submitted to GenBank and were assigned accession numbers M64557 through M64566. The sequence of the TEPC 1017 switch recombination site, which was previously published (17), has accession number K02150.

RESULTS

RNA analysis. RNAs from the nine IgD-secreting hybridomas and TEPC 1017 were analyzed by Northern (RNA) blots hybridized with probes for the different δ exons (Fig. 2). Hybridization to the C_δ probe looked identical to that to the $C_{\delta 3}$ probe and is not shown. The data are summarized in Table 2. Six of the hybridomas had δ mRNA bands of the same sizes as those seen in TEPC 1017 (45) and normal spleen (15): 1.75 and 3.2 kb ($\delta_{secreted}$ mRNA) and 2.1 and 2.9 kb ($\delta_{membrane}$ mRNA). RNA from the other three hybridomas, KWD1, KWD6, and 2C8, yielded bands smaller than those of TEPC 1017, and none of them hybridized to the $C_{\delta 1}$ probe. KWD1, as previously reported (43), and 2C8 RNAs had C_δ-hybridizing bands approximately 250 bases shorter than TEPC 1017 RNA. KWD6 RNA had C_δ-hybridizing bands about 100 bases shorter still and, unlike KWD1 and 2C8, failed to hybridize to the $C_{\delta H}$ probe as well. All of the RNAs hybridized to the $C_{\delta 3}$ probe. Deletion of C_δ domains occurs in our panel of hybridomas at a frequency similar to that suggested by a Northern blot of spleen RNA (see Fig. 8 in reference 43). This implies that the deletions of C_δ domains in these three hybridomas had occurred in the spleen and not during the generation of the hybridomas.

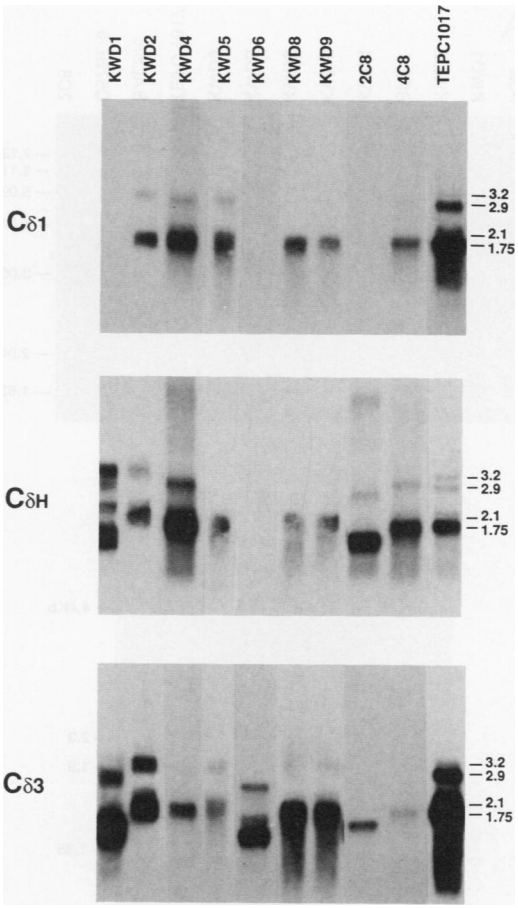

FIG. 2. RNA blots of IgD-secreting hybridomas. The RNAs were poly(A) enriched except for KWD5, KWD8, and KWD9. Probes are indicated on the left, and sizes (in kilobases) of the RNA bands of TEPC 1017 are indicated on the right. The panels are composites of two Northern blots which were aligned by normalization to the migration of TEPC 1017 bands.

TABLE 2. Hybridization of probes for C_δ exons to RNAs of IgD-secreting hybridomas

Hybridoma	Hybridization[a] with probe:		
	$C_{\delta 1}$	$C_{\delta H}$	$C_{\delta 3}$
KWD1	−	+	+
KWD2	+	+	+
KWD4	+	+	+
KWD5	+	+	+
KWD6	−	−	+
KWD8	+	+	+
KWD9	+	+	+
2C8	−	+	+
4C8	+	+	+

[a] +, hybridized to Northern blot RNA; −, failed to hybridize to Northern blot RNA.

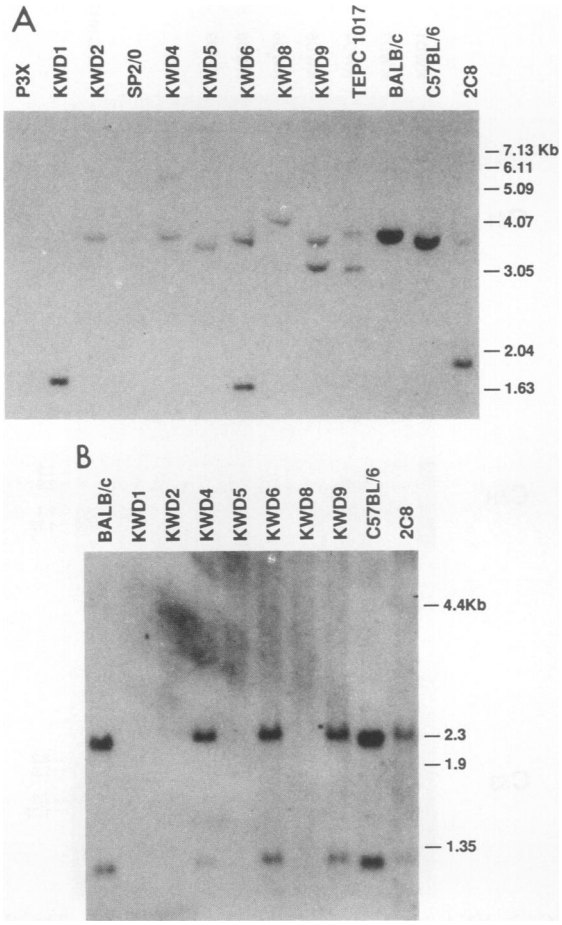

FIG. 3. Southern blots of *Hin*dIII-digested DNA from normal BALB/c and C57BL/6 tissues, IgD-secreting hybridomas, and fusion partners P3X and SP2/0. (A) Hybridization to the C_δ probe; (B) hybridization to the C_μ probe. Locations of size markers are marked at the right.

Southern blot analysis. The RNA data suggested that switch-related germ line deletions would have their 3′ ends between the $\mu_{membrane}$ exon and the $C_{\delta 3}$ exon. We expected that switch recombination would result in rearrangements of the 3.8-kb *Hin*dIII fragment, which contains most of the μ-δ intron and nearly all the C_δ sequences, as was the case for TEPC 1017 and TEPC 1033 (6, 17). This hypothesis was correct except for KWD2 and KWD5 (see below).

Blots of *Hin*dIII-digested DNA from each of the IgD-secreting hybridomas were hybridized to C_δ and C_μ cDNA probes (Fig. 3), and the data are summarized in Table 3. Since the P3X and Sp2/0 fusion partners lack the μ-δ locus (see our Fig. 3 and Fig. 9 in reference 43), any hybridoma bands hybridizing to the C_μ and C_δ probes must come from the spleen cells of the anti-IgD-treated mice. One of the nine hybridoma lines, 4C8, lost the C_δ locus, presumably by loss of chromosome 12, after RNA had been prepared and before DNA was prepared. Thus, it was not possible to determine the recombination site in 4C8.

Of the eight remaining hybridoma DNAs, all but KWD2 had rearranged the germ line 3.8-kb *Hin*dIII fragment (Table 3). It was clear that KWD2 had undergone a switchlike rearrangement upstream of the δ-hybridizing, germ line *Hin*dIII fragment, because there were no μ-hybridizing

TABLE 3. Hybridization of μ and δ probes to hybridoma DNAs[a]

Hybridoma	3.8-kb germ line *Hin*dIII δ band	Rearranged *Hin*dIII δ band	2.2- and 1.2-kb germ line *Hin*dIII C_μ bands
KWD1	−	1.8 kb	−
KWD2	+	−	−
KWD4	+	6.0 kb	+
KWD5	−	3.6 kb	−
KWD6	+	1.7 kb	+
KWD8	−	4.3 kb	−
KWD9	+	3.2 kb	+
2C8	+	1.9 kb	+

[a] +, band is present; −, band is absent.

restriction fragments. In addition to a rearranged C_δ *Hin*dIII fragment, KWD4, KWD6, KWD9, and 2C8 had the 3.8-kb germ line *Hin*dIII, δ-hybridizing fragment and the germ line *Hin*dIII, μ-hybridizing fragment (Fig. 3), indicating the presence of at least two chromosomes 12, one with a germ line configuration and one with a rearranged configuration. The other four hybridomas lacked the C_μ locus altogether. Thus, all eight of our IgD-secreting hybridomas had a chromosome which had deleted the C_μ locus, as had been seen in all previously studied murine, IgD-secreting plasmacytomas and hybridomas.

Recombination site sequences. Recombination sites were sequenced from oligonucleotide primers (Table 1) by using as templates either cloned *Hin*dIII fragments from KWD4, KWD8, and 2C8 or fragments generated by PCR on DNA from the remaining cell lines. Comparison of these sequences with germ line sequences revealed the locations of the recombination sites. Sequences of the switch recombination junctions of IgD-secreting cell lines are shown in Fig. 4, and the extents and locations of the deletions are shown graphically in Fig. 5. In nine cases there were between one and five bases (boxed in Fig. 4A) at the recombination junction which are present in the germ line at both the 5′ and 3′ breakpoints. The recombinations could have taken place at any of these overlapping nucleotides, so these small homologies between the 5′ and 3′ recombination-deletion breakpoints leave the exact site of the recombination deletion ambiguous.

(i) KWD1. The sequence across the recombination site demonstrated that the loss of expression at the RNA and protein levels was due to loss of the $C_{\delta 1}$ domain and not to aberrant RNA splicing.

(ii) KWD2. The recombination deleted nearly 11 kb from the germ line. The sequence of the recombination site confirmed the Southern blot data that the 3′ end of the switch was upstream of the germ line 3.8-kb *Hin*dIII fragment.

(iii) KWD4. The 6.0-kb *Hin*dIII fragment containing the KWD4 recombination site cloned in Charon 27 included about 3 kb of the S_μ-associated tandem repeat. This region is notoriously unstable when cloned in *Escherichia coli* (39), and in our hands the sizes of the clones varied by 2 kb from one isolate to another. Nevertheless, they all had the same flanking *Hin*dIII sites, and they all had the same 3′ recombination site just 5′ to the *Eco*RI site in the μ-δ intron, 2,702 bp upstream of the *Hin*dIII site in the $C_{\delta 3}$ exon. In all cases, the DNA 5′ of the *Eco*RI site is from the S_μ tandem repeat area (nearly exact repeats of GAGCT and GGGGT in an overall ratio of 3:1), and the 5′ breakpoint was calculated to lie about 3 kb into the tandem repeat area. The one-base homology between the two ends of the switch recombina-

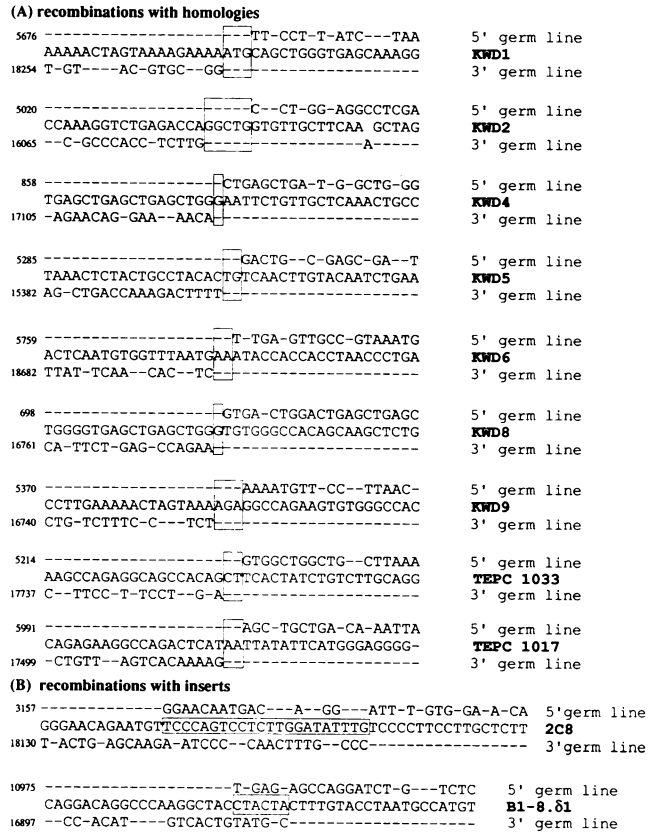

FIG. 4. Sequences of δ switch recombination junctions. The germ line sequences around the 5′ and 3′ recombination sites are above and below, respectively, the junction sequences from the indicated cell line. Dashes indicate identity to the hybridoma or plasmacytoma sequence; a space indicates deletion of a base. The initial germ line nucleotides are numbered according to the 28.2-kb sequence, SGENOME5, of Philip Tucker except for the tandem repeat sequences for KWD4 and KWD8, which were from Wesley Dunnick (GenBank accession number M28469). (A) Switch junctions with homologies at the recombination breakpoints. Boxes enclose bases that are identical at both breakpoints of the deletion. Note that KWD2 is missing a base that is in the germ line sequence of the 3′ recombination site. The germ line sequences around the 5′ recombination sites of KWD4 and KWD8 are not necessarily correct because the germ line sequence of the S_μ tandem repeat area is uncertain. Those displayed were chosen because they match the portions of the hybridoma sequences shown here. (B) Switch junctions with insert DNA. Boxes enclose the inserted bases.

tion-deletion indicated in Fig. 4 is uncertain because the precise sequence of the germ line is uncertain in this area.

(iv) KWD5. Two rearrangements were found in the μ-δ locus (Fig. 5): the switch recombination, and a small deletion and insertion in the μ-δ intron. The switch recombination junction was found by sequencing a PCR-generated fragment. The second rearrangement was found in the 3.6-kb δ-hybridizing *Hin*dIII fragment in Fig. 3, which was cloned and sequenced first because we thought that it contained the switch recombination site. However, the sequences of the *Hin*dIII sites flanking the cloned insert were the same as those of the germ line, 3.8-kb *Hin*dIII fragment. This was consistent with a 0.2-kb deletion, but the sequence of a PCR-generated fragment spanning the deletion showed a 394-bp deletion, including the *Eco*RI site, in the μ-δ intron of the germ line replaced by a 194-bp insertion. The insert, whose sequence will be reported elsewhere, was an incomplete V_H-D segment, inverted with respect to the J_H-C_δ locus. An *Eco*RI digestion of KWD5 DNA showed a 13.2-kb C_δ-hybridizing fragment instead of the 10.2-kb germ line fragment (data not shown), suggesting that this rearrangement was not an artifact produced by the PCR.

(v) KWD6. The sequence of a PCR-generated fragment showed that the recombination had deleted the $C_{\delta 1}$ and $C_{\delta H}$ exons as well as the C_μ gene. This confirmed the RNA and DNA blot data which showed that both the C_δ probe (Fig. 3) and the $C_{\delta H}$ probe (data not shown) hybridized with the rearranged 1.7-kb *Hin*dIII fragment but that the $C_{\delta H}$ probe did not hybridize with KWD6 RNA (Fig. 2). Thus, the 3′ breakpoint had to be among the 260 bases flanking the 3′ side of the $C_{\delta H}$ exon that are within the $C_{\delta H}$ probe.

(vi) KWD8. The clones of the 4.3-kb rearranged δ-hybridizing *Hin*dIII fragment were stable despite containing a portion of the S_μ tandem repeat region. A sequence primed by an oligonucleotide 3′ of the recombination site established that the downstream end of the deletion was 1 kb 5′ of the $C_{\delta 1}$ exon. The upstream recombination site was calculated from Southern blot data to be about 800 bases into the S_μ tandem repeat region. The sequence 5′ of the switch junction was consistent with the S_μ tandem repeat sequence (see results for KWD4 presented above) but did not precisely match any published S_μ repetitive sequence. The repeat from the germ line S_μ sequence in Fig. 4 was the most like that found 5′ of the switch junction in KWD8 and matched our sequence over the area displayed. There may be a one-base identity at the switch recombination endpoints as indicated in Fig. 4.

(vii) KWD9. The sequence through the recombination site showed that the 5′ recombination breakpoint was 6 bases from that of KWD1. The 3′ recombination breakpoint was 11 bases from that of KWD8.

(viii) 2C8. Partial sequence of the rearranged δ-hybridizing *Hin*dIII fragment subcloned in pGEM3Z showed that the 3′ end of recombination deletion was just a few bases beyond the $C_{\delta 1}$ domain. This explained how the $C_{\delta 1}$ probe could hybridize to the rearranged *Hin*dIII fragment (data not shown) but not to the RNA in Fig. 2. The 5′ recombination point lay within the pseudo-J_H exon between the J_{H3} and J_{H4} exons. This recombination was the farthest 5′ of all these hybridomas and had eliminated the upstream heavy-chain enhancer element (E_μ). 2C8 had a 22-base insert at the recombination site (shown in the box in Fig. 4B) instead of the short sequence homology between the flanks of the deleted areas seen in the KWD series. We tested the possibility that this insert was a sequence unique to C57BL/6 mice and therefore missing from the data base sequences which are principally from BALB/c mice. PCR with oligonucleotides flanking the pseudo-J_H exon generated fragments of equal size from DNA of both strains. The sequence of the C57BL/6 PCR fragment differed from that of the BALB/c fragment by only three point mutations in 300 bases around the 5′ switch recombination breakpoint in 2C8, ruling out a C57BL/6 polymorphism as the source of the insert.

(ix) B1-8.δ1. The partial sequence of a PCR-generated fragment containing the switch recombination site confirmed the restriction map of Sablitzky et al. (54). Like 2C8, the other C57BL/6 hybridoma, B1-8.δ1 had an insert (six bases) between the 5′ and 3′ recombination-deletion breakpoints instead of a short homology.

(x) Plasmacytomas. The switch-associated deletion in the BALB/c plasmacytoma TEPC 1033 was found to extend

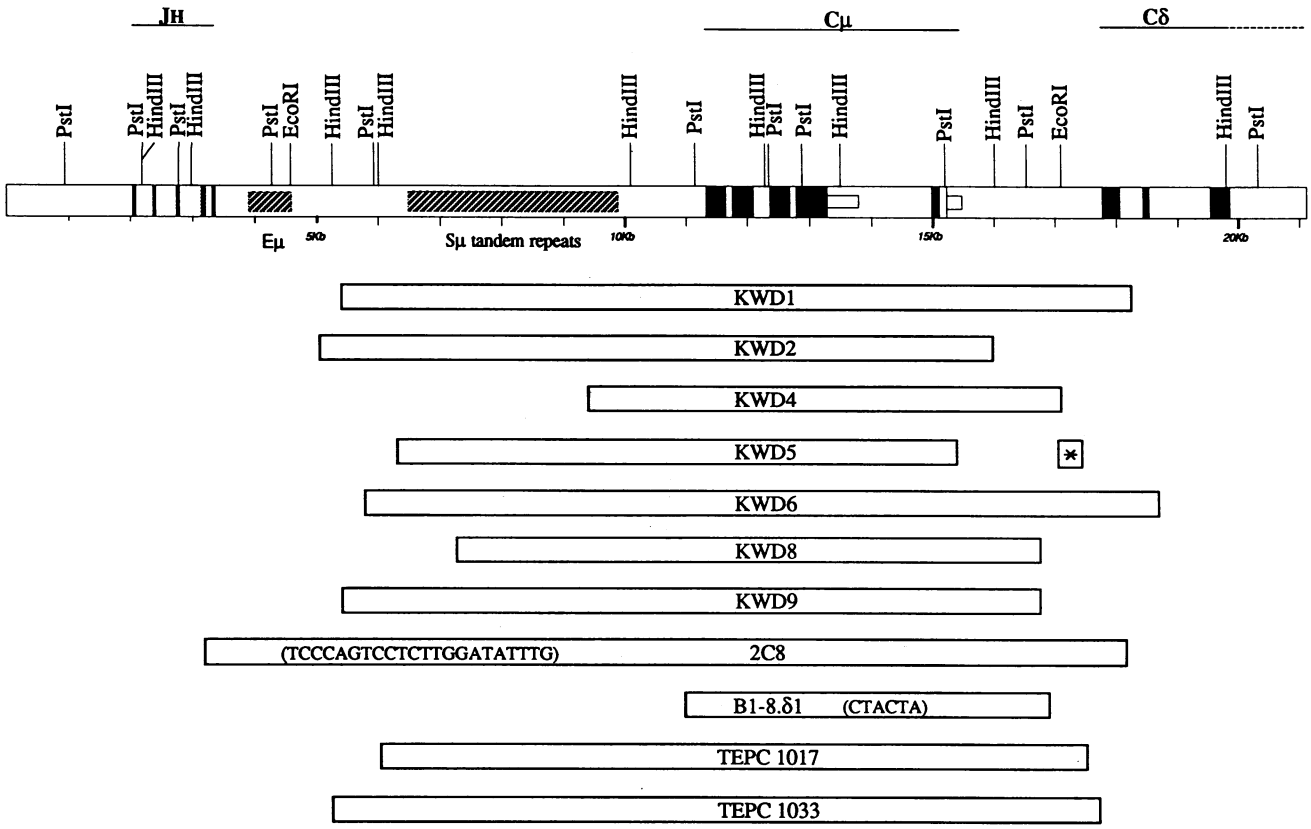

FIG. 5. Switch recombination-deletions from μ to δ. Boxes with the names of cell lines indicate the regions deleted by switch recombinations. The 394-bp deletion in the μ-δ intron of KWD5, with an asterisk to indicate a 194-bp insert, is shown on the same level as the switch deletion. Black boxes indicate coding sequences, and thin white boxes indicate untranslated but transcribed sequences. The gray box is the pseudo-J_H gene ($J_{H\Psi}$). The hatched areas indicate E_μ and the S_μ tandem repeats as labeled. The numbers are those of Philip Tucker's 28.2-kb sequence, SGENOME5.

from 1.2 kb upstream of the S_μ tandem repeat region to 20 bases upstream of the $C_{\delta 1}$ exon. The switch recombination junction sequence in TEPC 1017 has already been reported (17) and is included in Fig. 4 and 5 to complete the list of switch recombinations involved in IgD secretion.

DISCUSSION

Switch from IgM to IgD secretion occurs via a heavy-chain class switch recombination. IgD secretion is a rare phenomenon, particularly in the mouse, and thus it has been difficult to study. Administration of goat anti-mouse δ antibody stimulates IgD secretion (44) and has allowed us to generate a series of IgD-secreting hybridomas. Secretion of IgD in these hybridomas was accompanied by a somatic DNA rearrangement that deleted the C_μ locus in every case examined. It is important to know whether the secreted δ chain was encoded by the rearranged δ gene. In four of the hybridomas, the only δ locus present had been rearranged, and so must be responsible for the synthesis of the secreted IgD. In the other hybridomas, both a germ line and a rearranged μ-δ loci were present. In these cases, we cannot formally eliminate the possibility that the δ mRNA was synthesized from the unrearranged chromosome, but this seems unlikely since no IgD-secreting murine cell has only the germ line heavy-chain configuration.

In 3 of 10 hybridomas, one or more of the C_δ gene segments were deleted along with the C_μ locus. Apparently the deletion of $C_{\delta 1}$ does not necessarily abolish antigen binding, since KWD1 bound goat anti-mouse δ (43). However, loss of the C_δ domain(s) potentially could affect the function(s) of the secreted IgD, but this has not yet been tested.

Comparison of the DNA sequences of switch recombination sites in IgD-secreting cell lines with those of cells secreting other heavy-chain classes strongly suggests that the switch recombination resulting in IgD secretion utilizes the same mechanism as is used in switching to secretion of other heavy-chain classes. The nature of the junction sequences is similar in IgD and non-IgD switch recombinations. In published sequences of 24 productive murine heavy-chain switch recombination junctions (1, 10–13, 16, 21, 22, 25, 31, 40, 46, 47, 56, 60–63, 71, 74), 15 have 1 to 5 bases of overlapping homology between the two ends of the germ line deletions, 2 have longer homologies (6 and 11 bases), 7 have no homology, and 2 have short inserts (5 and 7 bases). (The remaining two involve areas of the germ line that have not been sequenced yet.) This is similar to the δ-secreting cell lines analyzed here, of which nine have a one- to five-base sequence homology in the germ line at the 5′ and 3′ ends of the deletions. The remaining two have 6- and 22-base inserts. Another similarity lies in the distribution of the 5′ ends of the recombination junctions (24) (Fig. 6) which are mostly upstream of the S_μ area of tandem repeats and only occasionally within the area of tandem repeats

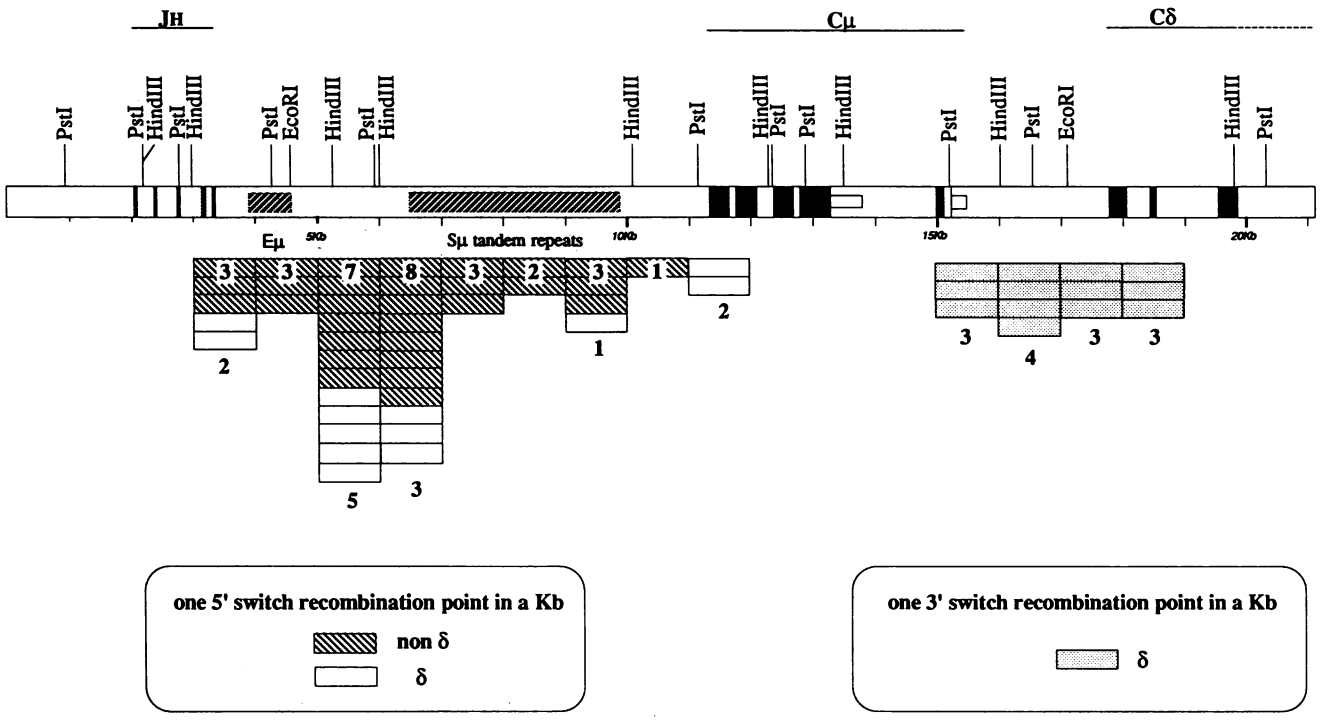

FIG. 6. Frequency distribution of switch recombination breakpoints in the μ-δ locus. Hatched rectangles represent 5′ switch recombinations to non-IgD isotypes. These were collected from published sequences (see text) and restriction maps (24) of productive murine switch recombinations reported in the literature. The δ switch recombination points are from this work and Klein et al. (26): white for 5′ and light gray for 3′ δ switch recombination points. There is one rectangle for each switch recombination breakpoint that occurs in the 1-kb region above it on the map.

itself. Although the 5′ recombination sites are distributed over nearly 8 kb of DNA upstream of the C_μ gene, most of the sites are located within the first 1,000 bases upstream of the S_μ tandem repeat area. It is a different story at the 3′ ends of recombination-deletions. Most, but not all, non-δ heavy-chain recombinations occurred within the 2- to 5-kb tandem repeat areas analogous to the S_μ tandem repeats. The mouse δ locus does not have such a repetitive area. Instead, the 3′ deletion endpoints in μ-to-δ switch recombinations were found within the 4 kb of DNA upstream of the $C_{\delta 3}$ exon, occasionally including the $C_{\delta 1}$ and $C_{\delta H}$ exons in the resulting deletion.

Switch recombinations are illegitimate. Early in the study of heavy-chain isotype switch recombination at the molecular level, "switch region" was coined as the term for a region responsible for class switch (22). Shortly afterward, tandem repeat sequences, with more or less frequent occurrence of two pentanucleotides, GAGCT and GGGGT, were found to be in the 5′ flank of each C_H gene except C_δ. "Switch region" became synonymous with the areas of tandem repeats and not with the actual sites of the switch recombinations, because signal sequences, like the heptamer-nonamer associated with joining V_H to D to J_H, could not be discerned around switch junctions and because the simple tandem repeats were the only recognizable structures outside of the heavy-chain exons. Homologous recombination between repetitive sequences with short stretches of homology was presumed to be the mechanism of heavy-chain switch recombination, just as it is the predominant mode of recombination in bacteria and yeasts (19).

The switch recombination to the C_δ gene in the mouse could not be homologous, since no tandem repeats are found in its 5′ flank. Instead, it has been considered an example of illegitimate, or nonhomologous, recombination (17). Random integration is one form of illegitimate recombination which occurs so readily in mammalian cells that transfected DNA, even with a very long (3- to 10-kbp) homology to its target, is 100 to 10,000 times more likely to be randomly integrated than to undergo homologous integration (29, 58). In fact, the sequences of the recombination junctions of the nine IgD-secreting hybridomas and of the two IgD-secreting plasmacytomas, as well as the sequences of the other switch recombination junctions, look very much like random integration sites (50): (i) there is little or no sequence identity found in the ends joined (42, 52) and (ii) switch recombination and random integration (51) both involve joining of DNA ends with occasional short inserts in the junctions. Thus, we would argue that the process of random integration is involved in all Ig heavy-chain class switch recombinations.

However, one characteristic of random integration, namely, wide, apparently random distribution in the genome, is not found in switch recombinations, since recombination of a V_H-D-J_H construct to a gene other than a C_H gene would not produce an Ig-secreting cell. Thus, the recombination process could have involved random sequences, but selection has expanded only Ig-producing cells, restricting the examples that can be studied and giving the appearance of nonrandom processes.

A switch recombination model. We favor a modified version of the looping-out and deletion model of switch recombination originally described by Jäck et al. (20) and extended by Petrini and Dunnick (47). In our working hypothesis, switching would be mediated by a switch complex which is

assembled in stages. In response to stimulation of mature B cells by interleukins and/or other T-cell- or macrophage-produced factors, nuclear proteins (73) would bind to the tandem repeat area of the μ locus. In response to other interleukins and other factors, the second portion of the switch complex would form near a downstream tandem repeat (switch) region (68), perhaps after the region is made accessible due to germ line (sterile) transcription (32, 36, 53). The two complexes would come together, endonuclease(s) would cut the two double helices, and ligation would occur, perhaps facilitated by enzymes in the complex. Under certain circumstances, a switch complex involving only the S_μ region would be formed on the IgH locus, and the second cut would occur in the 3′ flank of the C_μ locus, resulting in a switch to the δ locus. This region already would have an open chromatin configuration due to the usual read-through of the entire μ-δ locus during the pre-B-cell stage.

Usually a correct joint for a class switch is made. However, since there are several other cut ends nearby in the switch complex and since ligation randomly chooses which ends to join, it is not surprising that occasional mistakes would be made, such as those seen in this study which delete useful C_δ exons. Mistakes, such as inversions, have been documented in some heavy-chain switch recombinations (20). The IgH locus inversions and duplications that are attributed to sister chromatid exchange might also be mediated by this mechanism, since switch complexes can assemble on the C_μ of one chromosome and on a C_H on another chromosome (16).

Another possible utilization of this mechanism would lead to a translocation of the C_H locus to a transcriptionally active gene on a different chromosome (e.g., c-*myc*) if the two loci become physically close at a time when both are being actively transcribed. Recombinations to other loci also might occur, but they are not observed either because they are lethal or because they are undetectable except by accident, as in the case of ornithine decarboxylase, which was recombined head-to-head with the γ1 switch region (23).

Thus, the nonhomologous (illegitimate) recombination documented here for C_μ deletion in normal IgD-secreting spleen cells appears to be a general mechanism responsible for all heavy-chain class switching and, perhaps, for interchromosomal recombinations as well.

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