Rearrangements and Deletions of Immunoglobulin Heavy Chain Genes in the Double-Producing B Cell Lymphoma I.29

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The B cell lymphoma I.29 consists of a mixture of cells expressing membrane-bound immunoglobulin M (IgM) (λ) and IgA (λ) of identical idiotypes. Whereas most of the cells express either IgM or IgA alone, 1 to 5% of the cells in this tumor express IgM and IgA simultaneously within the cytoplasm and on the cell membrane (R. Sitia et al., *J. Immunol.* **127**:1388-1394, 1981; R. Sitia, unpublished data). When IgM⁺ cells are purified from the lymphoma and passaged in mice or cultured, a portion of the cells convert to IgA⁺. These properties suggest that some cells of the I.29 lymphoma may undergo immunoglobulin heavy chain switching, although it is also possible that the mixed population was derived by a prior switching event in a clone of cells. We performed Southern blotting experiments on genomic DNAs isolated from populations of I.29 cells containing variable proportions of IgM⁺ and IgA⁺ cells and on a number of cell lines derived from the lymphoma. The results were consistent with the deletion model for heavy chain switching, as the IgM⁺ cells contained rearranged μ genes and α genes in the germ line configuration on both the expressed and nonexpressed heavy chain chromosomes, whereas the IgA⁺ cells had deleted both μ genes and contained one rearranged and one germ line α gene. In addition, segments of DNA located within the intervening sequence 5' to the μ gene, near the site of switch recombination, were deleted from both the expressed and the nonexpressed chromosomes. Although μ genes were deleted from both chromosomes in the IgA⁺ cells, the sites of DNA recombination differed on the two chromosomes. On the expressed chromosome, S_{μ} sequences were recombined with S_{α} sequences, whereas on the nonexpressed chromosome, S_{μ} sequences were recombined with $S_{\gamma 3}$ sequences.

During differentiation, a clone of B lymphocytes shifts from the synthesis of immunoglobulin M (IgM) to the synthesis of other classes of immunoglobulins while continuing to express the same variable (V_H) domain (1, 10, 19, 29, 30). As the class of immunoglobulin is determined by the constant region of the immunoglobulin heavy (H) chain (C_H) region, the B cell must switch from the expression of the C_{μ} gene to the expression of other C_H genes. By examining the DNA of myeloma and hybridoma cells, it has been concluded that H chain switching is effected by deletion of the C_H genes (and their flanking sequences) located between the expressed V_H - D - J_H gene and the newly expressed C_H gene (5, 8, 12, 12a, 14, 45).

Plasmacytoma and hybridoma cells are terminally differentiated cells which are generally not capable of H chain switching. Hence, compari-

sons of the H chain genes in myelomas and hybridomas expressing different H chains do not necessarily reflect precursor-product relationships, as additional changes could have occurred in the genomes of these cells since the switch occurred. Furthermore, it is not known whether the deletions which appear to effect the switch occur in a single step or whether intermediate deletions occur.

If H chain switching is effected by deletion, individual cells should only be able to sustain the synthesis of two different H chains with the identical V_H region by utilizing long-lived mRNA for one H chain until such stores of mRNA are exhausted. Indeed, individual cells have not been found to continuously synthesize two different H chains with the same V_H region, with the exception of μ - and δ -producing cells. The close proximity of the μ and δ genes appears

to allow transcription of a single large RNA which can be spliced in alternate ways (20, 21, 24).

The B cell lymphoma I.29, which is the subject of this report, consists of a mixture of cells expressing surface-associated IgM (λ) and IgA (λ) of identical H chain idiotypes (38). Most of the cells express either IgM (λ) or IgA (λ) alone, but 1 to 5% of the cells express both cytoplasmic and membrane-bound IgM and IgA simultaneously (38; R. Sitia, unpublished data). When IgM⁺ cells were purified from the tumor and cultured *in vitro*, they converted entirely to IgA⁺ in approximately 11 days (38). Similarly, IgM⁺ cells, on five consecutive passages in mice, showed a gradual change to IgA⁺ cells until the proportions of IgM⁺ and IgA⁺ cells approximated those of the unsorted tumor (see Table 1). These properties suggest that I.29 may be a model for cells undergoing H chain switching. We have as yet to prove that switching occurs in this system, as efforts to clone IgM⁺ cells in culture or *in vivo* have not been successful, and therefore it is possible that the few IgA⁺ cells which contaminate the IgM⁺ cell preparations could overgrow the IgM⁺ cells to produce the observed change to IgA⁺. The presence of double-producing cells, however, argues for the occurrence of active switching.

Since the IgM⁺ and IgA⁺ cells express the same idiootype and, apparently, the same germ line V_H gene and also contain the same rearrangement of a nonexpressed C_k gene (38; see below), the various cells of the I.29 lymphoma were probably derived from a single clone of cells. Hence, this lymphoma provides a system which can be used to examine the structure of immunoglobulin genes before and after switching in a single clone of cells. In this report, we examine the structure of immunoglobulin genes by restriction enzyme mapping of genomic DNAs isolated from a number of cloned cell lines and cell populations derived from the I.29 lymphoma. DNA deletions are clearly responsible for the switch in expression of C_H genes in the I.29 lymphoma.

MATERIALS AND METHODS

Cell lines. The B cell lymphoma I.29 arose spontaneously in the I/St mouse strain at the Sloan-Kettering Institute in 1961 (34, 35). The leukemia is maintained by intraperitoneal passage in I/St mice or in (BALB/c $\times$ I/St)_F₁ mice.

The cell lines used in these experiments were derived from the I.29 tumor as follows. The IgM-secreting hybridoma ID43 and the IgA-secreting hybridoma ID150 were derived by fusion of I.29 cells with the nonsecreting myeloma cell line P3-NS.1-Ag-8 (41). These hybridomas express only IgM or IgA, respectively. No double-producing hybridomas were obtained. Two cloned cell lines which express mem-

brane-bound IgA, BFO1.1 and BFO.3, were derived by adaptation of I.29 cells to culture (38). A noncloned cell line, IgM.S, was derived by culturing 10³ IgM⁺ cells (obtained by sorting I.29 cells in a fluorescence-activated cell sorter [FACS]) in Millipore filter chambers within the peritoneal cavities of I/St mice. Several chambers were inoculated, but only one chamber yielded I.29 cells, and these all expressed IgM of the I.29 idiootype. Cells were cultured in RPMI 1640 medium supplemented with 10% newborn calf serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Immunofluorescence and cell sorting. Production, purification, and characterization of most antibodies used in this study have been described in previous reports (38, 41). They are briefly characterized as follows. Antibody to mouse IgM was the culture fluid of the hybridoma R33/24, which secreted rat antibody to mouse μ chains (kindly provided by Gunter J. Hammerling, Heidelberg, West Germany). Antibody to IgA was prepared from rabbit antiserum to IgA (from ID150) by chromatography on solid-state ID150 protein, followed by absorptions with myeloma protein HOPC1 [IgG (λ)] to remove anti- λ and with hybridoma ID43 to remove anti- κ and anti-I.29 idiotypes. Anti-I.29 idiootype was a mouse monoclonal antibody derived from hybridoma T10/219 (42). Two other idiootype-specific antisera were prepared from rabbit antisera to hybridomas ID150 and ID43 by exhaustive absorption with normal mouse immunoglobulins and then with either IgA of MOPC315 or IgM of TEPC183, respectively.

For all immunofluorescence experiments, I.29 tumor cells were taken from ascites after passage for 4 to 8 days in I/St or (BALB/c $\times$ I/St)_F₁ mice and cultured for 2 h or overnight to remove cytophilic antibodies. Cells were labeled in suspension in a two-step procedure involving incubation with anti- α , anti- μ , or anti-idiootype antibodies, followed by incubation with fluorescein-labeled, affinity-purified goat anti-rabbit IgG, rabbit anti-rat IgG, or rabbit anti-mouse IgG/Fc, respectively. A directly fluoresceinated anti- μ from hybridoma R33/24 was used in some experiments. A detailed account of serological and immunochemical characterizations of the I.29 tumor and derivative cell lines has been previously reported (38).

Cell-sorting experiments were performed with the FACS IV. For enriching μ ⁺ cells, 25% of the brightest-stained cells were collected. Analysis of these cells immediately after collection showed purities of 95 to 98%. Preparations of lower purity were rejected. The immunoglobulins expressed in I.29 tumor cells, cell lines, and sorted cells were evaluated by immunofluorescence microscopy; the results concurred with published data (38).

Karyotype analysis. Karyotype analyses were performed by Giemsa band staining after denaturation of the chromosome, using a slight modification of the trypsin denaturation technique (37). The chromosomes were identified by referring to published studies (7, 25, 28).

Restriction enzyme digestion, gel electrophoresis, and DNA blotting. Samples (21 µg) of high-molecular-weight DNA, isolated as described previously (3), were digested with the indicated restriction enzymes (purchased from New England Biolabs, Bethesda Research Laboratories, or Boehringer Mannheim Corp.).

To monitor the extent of digestion, 5% of the reaction mixture was added to 0.3 μ g of pBR322 or λ bacteriophage DNA and incubated along with the main reaction (13). The test samples were analyzed by electrophoresis in a 0.8% agarose gel in 40 mM Tris-hydrochloride–20 mM acetate–18 mM NaCl–2 mM disodium EDTA (pH 8.1) (TEA-Na). When the digestion was complete, the DNA of the main reaction was precipitated with ethanol, and 10- μ g portions were electrophoresed on 6-mm-thick 0.7% agarose gels in TEA-Na.

DNA was transferred to nitrocellulose filters (39). The blots were baked, preannealed for 6 to 20 h, and annealed for 3 days at 41°C with 32 P-labeled restriction fragments in a solution containing 50% deionized formamide, 450 mM NaCl, 45 mM sodium citrate, 0.3 mM disodium EDTA, 1 mg of yeast RNA per ml, 100 μ g of *Escherichia coli* DNA per ml, and 0.02% each of bovine serum albumin, Ficoll, and polyvinyl pyrrolidone. The blots were washed in 0.1% sodium dodecyl sulfate–15 mM NaCl–0.1 mM disodium EDTA–1 mM phosphate (pH 6.8) at 50 to 52°C and were exposed at –80°C to X-ray film (Kodak XR), using a Dupont Lightning Plus intensifying screen. If the blot was to be annealed with another probe, the first probe was removed by incubation of the blot at 68°C for 15 to 30 min in preannealing mix.

The restriction maps were determined by annealing the indicated probes to numerous Southern blots of single and double enzyme digests of DNAs from I.29 cells and from I/St and BALB/c liver cells and by a comparison of our data with published data on BALB/c μ and α genes (20, 23, 33). The restriction fragments obtained from the germ line J_H - C_μ loci in I/St and BALB/c mice were identical, except for those which included the length variation region between the two *Hind*III sites located 1.5 and 5 kilobases (kb) 5' to the C_μ gene. The restriction enzyme sites which were altered in the rearranged μ genes could be located relative to the sites in germ line DNA by using the various probes indicated. The α gene in I strain mice is of a different allotype (41, 42) and has some restriction sites which differ from the α gene in BALB/c mice; the sites for *Bgl*III and *Kpn*I in the α gene were located by assuming that the V_H genes expressed with C_μ and C_α were identical. The other sites were mapped as described for the J_H - C_μ loci. As we could locate only those restriction sites which produced fragments that annealed with the indicated probes, some sites for the enzymes we used are missing from our maps. The deletion events occurring within the intervening sequence in the expressed and nonexpressed chromosomes could be distinguished by comparing the bands detected by pj_0 and pj_{11} and also by comparing the bands detected in IgM.1P, IgM.S, and BFO cells, as these three cells contained different forms of the nonexpressed J_H allele.

Plasmid purification and preparation of probes for hybridization. Plasmids were isolated from cleared lysates (by a modification of a previously described method [4]) by phenol-chloroform extraction at pH 4.0 (46) and gel filtration on Sepharose 4B. Hybridization probes were prepared by isolation of restriction fragments from the plasmids. DNA probes were nick-translated (31) to a specific activity of approximately 4×10^8 cpm/ μ g, using [α - 32 P]TTP at 3,000 Ci/mmol. All bacteria containing recombinant DNA were contained

at the P1/EK1 level as specified by the National Institutes of Health Guidelines for Recombinant DNA Research.

Hybridization probes. The hybridization probe for C_μ was a *Bgl*III/*Hind*III fragment isolated from the plasmid $p\mu(3741)^9$ which contained cDNA coding for C_μ (23). Figure 1A shows the sequences coding for the C_μ probe (labeled μ) on the restriction map of the I strain genomic μ gene. Probes for J_H genes were from the plasmid clones pj_0 and pj_{11} , which contain BALB/c genomic DNA sequences coding for the J_{H1} - J_{H2} (J_{H1-2}) and the J_{H3} - J_{H4} (J_{H3-4}) gene segments, respectively (23; Fig. 1A). $pM2$ -20 contained a *Hind*III fragment of BALB/c genomic DNA sequences from the region 5' to the C_μ gene, which included the switch recombination (S_μ) sequences (23). $pM2$ -5B contained a *Hind*III insert of BALB/c genomic sequences which included the 5' portion of the C_μ gene and 5' flanking sequences (23). The sequences contained in the genomic DNA clones are indicated on the restriction map of the I strain germ line J_H - C_μ gene (Fig. 1A). The probe for C_α was an *Msp*I fragment containing nearly the entire C_α sequence and isolated from the cDNA plasmid $p\alpha(J558)^{13}$ (23). The probe for $C\gamma 2b$ was a *Pst*I/*Kpn*I fragment isolated from the plasmid $py2b(11)^7$ (36). The probe pBR1.4 contained BALB/c genomic DNA sequences from the $S\gamma 2b$ region located 5' to $C\gamma 2b$. The sequences used as the $S\gamma 2b$ probe are indicated on the map of the germ line $C\gamma 2b$ gene (18; see Fig. 4a). The probe for $C\gamma 3$ was a 6.6-kb *Hind*III fragment containing the BALB/c germ line $C\gamma 3$ gene and flanking sequences prepared from the recombinant λ phage SL51 (K. B. Marcu, L. W. Stanton, R. Lang, and M. Diamond, unpublished data). The probe for immunoglobulin λ chain genes was an *Hha*I fragment containing cDNA coding for the entire λ chain of the myeloma H2020 from the plasmid A12, which was similar to the plasmid B1 described previously (2). The probe for immunoglobulin $C\kappa$ sequences was a 730-base pair fragment containing cDNA sequences coding for $C\kappa$ and a small portion of the V region of the MOPC384 κ chain isolated from the plasmid $pM384\kappa$ by *Hind*III digestion (40).

RESULTS

Restriction enzyme mapping of immunoglobulin genes in I.29 cell lines: deletion of μ genes and rearrangement of α genes. To study the DNA rearrangements and deletions which accompany H chain switching in the I.29 cell line, DNAs from several different cell lines and cell populations prepared from the I.29 lymphoma were digested with restriction enzymes and the DNA fragments were analyzed by DNA blotting (41). The blots were annealed with 32 P-labeled cloned cDNAs or with cloned DNA sequences from the BALB/c genome containing immunoglobulin genes and their flanking sequences. The nucleotide sequences complementary to the probes used in the first series of experiments are indicated on the restriction maps of germ line μ and α genes of I strain mice (Fig. 1A); the probes are further described in Materials and Methods.

FIG. 1. Restriction enzyme maps of μ and α genes in I.29 and I strain liver cells. (A) Maps of the germ line μ and α genes in liver cells and of the expressed μ and α genes in I.29 cells. (B) Maps of the three fragments from the nonexpressed J_H -C μ allele in I.29 cells compared with the map of the fragment produced by joining of the D_{Q52} and J_{H2} genes described in references 17 and 32. The nucleotide sequences encoding the probes used to detect these fragments are indicated below the maps of the germline μ and α genes. S_μ and S_α indicate the regions in which switch recombination between the expressed μ and germ line α genes, respectively, appears to occur in I.29 cells. The site of recombination is within the 2.0-kb region indicated by filled boxes on the expressed α gene. Δ -0.5, Δ -1.0, and Δ -2.0 kb indicate sites where DNA sequences have been deleted from the intervening sequence between J_{H4} and C μ . The thick horizontal lines and bars identified by V, J, and C indicate coding sequences or potential coding sequences. DNA sequences which originate from different sites in the germ line DNA are indicated by different types of horizontal lines and bars. The DNA sequence, indicated by the shaded bar in (B-3), is derived from the C γ 3 gene. The site of recombination with the S_μ sequences is located 4 to 6 kb 5' to the C γ 3 gene. The map of the D_{Q52} - J_{H2} fragment was redrawn from the map of the cloned DNA fragment analyzed by Sakano et al. (32) and Kurosawa et al. (17), who did not map the *Sac*I or *Pst*I sites in their clone. The restriction enzyme sites identified are: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sac*I; and X, *Xba*I.

DNA was prepared from populations of I.29 cells obtained by sorting the I.29 μ^+ cells on the FACS, using a monoclonal rat anti-mouse μ antibody and passing these cells in mice (Table 1). These cell populations were termed IgM.1P, IgM.2P, etc., the number indicating the number of passages in mice after sorting. The proportions of cells expressing μ and α were determined by immunofluorescence. The ratio of α^+ to μ^+ cells generally increased during subsequent passages, although there was vari-

ability among different experiments (Table 1). DNA was also prepared from the following cloned cell lines expressing either μ or α : IgA⁺ cells obtained by cloning the tumor in vitro (BFO.3 and BFO1.1) (38), an IgM hybridoma (ID43), and an IgA hybridoma (ID150) derived from the fusion of I.29 tumor cells with the nonsecreting myeloma cell line P3-NS.1-Ag-8 (41). Thus far, no IgM cell line has been cloned from the I.29 lymphoma, as culture conditions conducive to growth of the IgM⁺ cells in vitro

TABLE 1. Cell populations and cell lines derived from the I.29 lymphoma

Designation	Assay	% Positive		
		μ ⁺	α ⁺	Idio- type ⁺
Cell populations ^a				
IgM.1P	FACS	99 ^b	37	97
IgM.2P	FACS	41	54	76
IgM.3P (prepn 1) ^c	FACS	70	40	NT ^d
IgM.3P (prepn 2)	FACS	41	51	>90
IgM.4P	FACS	40	60	NT
IgM.5P	FACS	17	77	93
Cell lines				
ID43 (IgM hybridoma)	IFA	100	0	100
ID150 (IgA hybridoma)	IFA	0	100	100
BFO1.1, BFO.3	IFA	0	100	100
IgM.S	FACS, IFA	>95	0	>95

^a Mixed cell populations were obtained by sorting I.29 cells on the FACS and passaging the cells intraperitoneally in I/St or in (I/St $\times$ BALB/c)F₁ mice. The cell populations indicated here were used in the restriction mapping experiments but were not all from the same FACS separation.

^b The percentage of μ^+ cells in this preparation is inconsistent with those of two other preparations of IgM.1P cells, which were 70% μ^+ and 30% α^+ in preparation 1 and 45% μ^+ and 44% α^+ in preparation 2. We believe that this is due to a problem with the FACS assay in the experiment shown above.

^c The IgM.3P cells used in these experiments were derived from two independent sorting experiments (referred to as preparations 1 and 2 in the text).

^d NT, Not tested.

have not been found. However, a line of IgM⁺ cells (IgM.S) which grows only in vivo without, as determined so far, switching to IgA expression, was derived by growing IgM⁺ cells (obtained from the FACS) in Millipore chambers in mice (U. Hammerling and R. Chua, unpublished data). Livers of I/St mice were used as a source of germ line DNA since immunoglobulin genes in liver cells have been shown to be in the same context as in sperm or embryo cells (8, 15, 22, 44).

The genomic DNAs were digested with a number of restriction enzymes (as indicated in Fig. 1) in single and double enzyme digests. The fragments detected when DNAs were digested with *Kpn*I and annealed with μ cDNA are shown in Fig. 2A. The maps of these DNA segments are shown in Fig. 1, and the derivation of these maps are described below and in Materials and Methods. As *Kpn* has a recognition site located 5' to the *J_H* genes in germ line DNA, joining of the *V_H*, *D*, and *J_H* gene segments altered the *Kpn* fragment that annealed with μ cDNA (Fig. 1A). The μ genes on both chromosomes were

rearranged in the IgM hybridoma and IgM.S cells, as these cells contained two *Kpn* fragments which both differed in size from the *Kpn* fragment in liver DNA (Fig. 2A). Note that the NS.1 plasma cells used for fusion to produce the hybridomas did not contain *C μ* genes (Fig. 2A, lane NS.1). From our extensive restriction mapping (see below and Fig. 1), we determined that the larger *Kpn* fragment (15.3 kb) in IgM⁺ cells contained the expressed μ gene and was produced by a recombination near the *J_{H4}* gene segment (Fig. 1A). The smaller *Kpn* fragment was produced by a recombination near the *J_{H2}* gene segment on the nonexpressed chromosome. In addition, DNA sequences were deleted from the intervening sequence between the *J_{H4}* and *C μ* genes at both *J_H-C μ* alleles (Fig. 1; see below). The last two lanes in Fig. 2A (BFO1.1 and IgA hybridoma) show that, in the BFO cells (cloned IgA⁺ cells) and IgA hybridoma cells, the *C μ* genes were deleted from the expressed and nonexpressed chromosome. IgA⁺ cells obtained by sorting I.29 tumor cells on the FACS also did not contain any *C μ* genes (data not shown).

When DNAs from cells which had been enriched for μ^+ on the FACS and subsequently passaged one to five times in mice were annealed with μ cDNA, we found that, as the proportion of IgA⁺ cells increased, the intensity of the μ bands generally decreased (compare lanes containing DNA from IgM.S, IgM.3P [preparations 1 and 2], IgM.4P, and IgM.5P cells [0, 40, 51, 60, and 71% α^+ , respectively] in Fig. 2A; the amount of IgM.1P DNA loaded onto the gel was less than that of the other DNAs, so the intensity of the bands in IgM.1P DNA cannot be compared with those of the other DNAs). The results shown in Fig. 2A demonstrated that μ genes are lost from both chromosomes as the proportion of the IgA⁺ relative to IgM⁺ cells increases.

A third μ fragment of 11 kb was detected in *Kpn* digests of DNA from the IgM.1P and IgM.3P (preparation 1) cell populations, in addition to the 15.3-kb fragment derived from the expressed chromosome and the 10-kb fragment derived from the nonexpressed chromosome. The presence of three μ fragments was not due to polyploidy of the I.29 cells, as karyotype analyses of I.29 tumor cells, IgM.S cells, and BFO.3 cells indicated that each of these cells contained an average of two H chain chromosomes (chromosome 12) and an average of 41 chromosomes altogether (data not shown). Furthermore, the cloned cell lines contained only one fragment from the expressed H chain allele and one fragment from the nonexpressed allele. The restriction maps (Fig. 1B; see below) indicated that the 11-kb *Kpn* fragment was derived from the nonexpressed allele. As the 11-kb frag-

FIG. 2. Southern blots of *KpnI* digests of genomic DNAs from I.29 cell lines. Samples (10 μ g) of genomic DNAs from I.29 cells described in Table 1 were digested with *KpnI*, electrophoresed on 0.7% agarose gels, blotted to nitrocellulose, and annealed with 32 P-labeled probes. (A) μ cDNA; (B) α cDNA; (C) *Bam*HI fragment from *pj*₀ containing the *J*_{H1} and *J*_{H2} gene segments; (D) *Bam*HI/*Eco*RI fragment from *pj*₁₁ containing the *J*_{H3} and *J*_{H4} gene segments. The sizes of the fragments indicated on the right side of the blots were determined relative to the sizes of the *Hind*III fragments of λ bacteriophage DNA, which had been labeled with [γ - 32 P]ATP by T4 polynucleotide kinase and electrophoresed in a parallel lane. The lanes in (A) and (D) of DNA from IgM.1P cells contained much less DNA than the other lanes; hence, the intensity of the bands in these lanes cannot be compared with the intensity of the bands in other lanes. NS.1 indicates P3-NS.1-Ag-8.

ment was not detected in DNA from late passages (IgM.4P and IgM.5P), this fragment may be a precursor to the 10-kb fragment. The data from the restriction maps support this possibility (compare Fig. 1B-1 and 1B-2). It is also possible that the 11-kb fragment is not a precursor to the 10-kb fragment but was simply present in the population of IgM⁺ cells selected by the FACS and that these cells were overgrown by cells containing the 10-kb fragment during passage of the tumor. The 11-kb fragment was found only in the cell populations obtained by sorting the tumor cells and passaging them in vivo. Both the IgM hybridoma we examined and the IgM.S cells contained only the 10-kb *Kpn* fragment from the nonexpressed allele (Fig. 2A).

The *Kpn* fragments detected with 32 P-labeled α cDNA are shown in Fig. 2B. The IgM hybridoma and IgM.S cells contained only the 16.5-kb germ line α fragment. (The large fragment [40 to 60 kb] detected in DNA from the IgM hybridoma contained α sequences contributed by the P3-NS.1-Ag-8 genome.) As the FACS-sorted IgM⁺ cells were passaged in mice and the proportion of IgA⁺ cells increased, a rearranged α fragment (25.5 kb) appeared and subsequently increased in intensity. In BFO cells, the rearranged α fragment was of an intensity similar to that of the germ line α fragment. The IgA hybridoma and IgA cells obtained by sorting the I.29 tumor cells on the FACS also appeared to contain an equimolar amount of the germ line and rearranged α gene fragments (data not shown).

I.29 DNAs were annealed with two different cloned BALB/c genomic DNA sequences containing either the *J*_{H1} and *J*_{H2} genes (*pj*₀) or the *J*_{H3} and *J*_{H4} genes (*pj*₁₁) (23). The DNA segments contained in these probes are shown in Fig. 1A. The *Kpn* fragments detected with the *J*_{H3-4} probe are shown in Fig. 2D. The *J*_{H3-4} probe annealed with the same 13.6-kb fragment from liver DNA as did the μ cDNA probe. The *J*_{H3-4} probe also annealed with the *Kpn* fragments containing the expressed μ (15.3 kb) and α (25.5 kb) genes and with the two μ fragments from the nonexpressed H chain allele (10 and 11 kb). In addition, both *J*_H probes (*pj*₀ and *pj*₁₁) detected a 7.5-kb *Kpn* fragment which did not anneal with the μ or α probes (compare Fig. 2A and B with Fig. 2C and D). The map of this fragment is shown in Fig. 1B-3. The 7.5-kb *Kpn* fragment appears to be derived from the 10-kb *Kpn* fragment or the 11-kb *Kpn* fragment, or both, from the nonexpressed *J*_H-C μ locus, since these three fragments had identical restriction maps on the 5' side of the *J*_H genes (Fig. 1B) and since the 7.5-kb band appeared to increase in intensity as the 11-kb fragment disappeared (Fig. 2D; compare lanes IgM.3P [preparation 1] and IgM.3P [preparation 2]). The 7.5-kb *J*_H fragment was absent from the IgM hybridoma and IgM.S cells and barely detectable in the IgM.1P population. The amount of this fragment increased when more IgA⁺ cells were present in the population, and in BFO cells, it was the only portion of the *J*_H-C μ locus from the nonexpressed chro-

mosome which remained. By annealing a cloned DNA sequence containing the germ line $\gamma 3$ (described in Materials and Methods) with blots of I.29 cell DNAs (data not shown), we determined that this fragment contains a rearranged $\gamma 3$ gene. The sequences coding for $C\gamma 3$ are located immediately 3' to the *Bam* site indicated on the stippled DNA sequence (Fig. 1B-3). This result has been confirmed by analysis of a recombinant λ bacteriophage containing an 8.5-kb *Bam* fragment from BFO cell DNA which contains J_H gene segments and $\gamma 3$ sequences (J. Stavnezer and S. Sirlin, unpublished data).

To summarize the results of mapping the nonexpressed H chain allele in I.29 cells, a recombination event appeared to have occurred at or near the J_{H2} gene, since the *HindIII*, *BglII*, and *PstI* sites between J_{H1} and J_{H2} were deleted (Fig. 1B). In addition, in cells which have switched from IgM to IgA synthesis, recombination events occurred in the intervening sequence between the J_{H4} and $C\mu$ genes which resulted in the deletion of the $C\mu$ gene from the nonexpressed allele and the joining of the J_H genes with sequences located 4 to 6 kb 5' to the $C\gamma 3$ gene. As the $\gamma 3$ switch recombination ($S\gamma 3$) sequences are located between 1.7 and 5 kb 5' to the $C\gamma 3$ gene (16, 18), this recombination was probably between $S\mu$ and $S\gamma 3$ sequences.

Identification of DNA fragments containing the expressed μ gene; mapping the expressed V_H gene. To determine which of the three μ fragments detected in IgM.1P cells contains the expressed μ gene, the restriction maps of the μ gene fragments and the rearranged α gene fragment were compared. Since the H chain idiotypes of the IgM and IgA synthesized in I.29 cells are identical, we expected that the restriction maps of the DNA sequences located 5' to the J_H genes in the rearranged α gene and in the expressed μ gene would be similar. The mapping data are consistent with this hypothesis. The J_{H3-4} probe annealed with the rearranged α gene, whereas the J_{H1-2} probe did not (Fig. 2C and D); hence, the expressed α gene has deleted the J_{H1} and J_{H2} gene segments. The larger of the two μ fragments (15.3 kb) in the IgM hybridoma and IgM.S cells also annealed with the J_{H3-4} probe, but not with the J_{H1-2} probe, suggesting that this fragment bears the expressed μ gene (Fig. 2C and D). The *HindIII* site located between J_{H3} and J_{H4} in germ line DNA is missing from the expressed μ and α genes (Fig. 1A); hence, the J_{H3} gene has probably been deleted, and J_{H4} is probably expressed in I.29 cells. Proof that J_{H1} and J_{H2} were deleted from the expressed μ and α genes was provided by the finding that the J_{H1-2} probe did not hybridize with blots of RNAs from various I.29 cell lines, whereas the J_{H3-4} probe hybridized with RNAs of the same size as those

detected by the μ and α probes (data not shown).

The restriction maps of the DNA sequences located 5' to the rearranged J_{H4} gene in the expressed μ and α genes were compared by digestion with restriction enzymes that cleave between J_{H4} and the $S\mu$ sequences. Figure 3A shows that the fragments produced by digestion with *EcoRI*, *SacI*, *PstI*, or *HindIII* or by both *HindIII* and *BamHI* together, which were detected by the J_{H3-4} probe, were identical in DNAs from all of the I.29 cell lines expressing IgM or IgA but differed from those in liver DNA. These restriction sites are mapped in Fig. 1A. We concluded that the V_H genes expressed with $C\mu$ and with $C\alpha$ in the I.29 cells are very similar.

Restriction maps of the fragments that annealed with the J_{H1-2} probe indicated that the nucleotide sequences located 5' to the J_H genes on the three J_H fragments from the nonexpressed allele are also identical (or very similar) in all of the I.29 cell lines (Fig. 1B and 3B).

Site of switch recombination in I.29 cells. The sites at which DNA recombination occurred during switching from μ to α in the I.29 cells are within the tandemly repeated *S* sequences identified in myeloma cells (9, 16, 18, 26, 33). The site within the $S\mu$ sequence at which recombination with the $S\alpha$ sequence occurred in I.29 cells [$S\mu(\alpha)$] was between the *HindIII* and *SacI* sites, which were located 2 and 4.5 kb, respectively, 5' to the $C\mu$ gene (Fig. 1A). The $S\mu(\alpha)$ sites in two IgA myelomas, T15 and M603, are located approximately 1 kb 5' to this *SacI* site (9). The switch site 5' to $C\alpha$ ($S\alpha$) was between the *PstI* and *SacI* sites located approximately 2 and 4 kb, respectively, 5' to the $C\alpha$ gene and indicated by filled boxes on the map of the expressed α gene (Fig. 1A).

If individual I.29 cells were actively switching, the sites of DNA recombination could be expected to differ among different cells, because the nucleotide sequences of the $S\mu$ and $S\alpha$ regions consist of tandem direct repeats extending over approximately a 4- and a 2.4-kb region of DNA, respectively, and because the sites of switch recombination differ among different myelomas (9, 16, 26, 27, 33). We attempted to detect heterogeneity in the $S\mu(\alpha)$ recombination site by comparing the sizes of *SacI* fragments containing the expressed α gene in three different cloned IgA cell lines, BFO.3, BFO1.1, and the IgA hybridoma. The two BFO cell lines were derived in the same experiment by seeding in separate wells I.29 tumor cells immediately after they were removed from a mouse (38); the IgA hybridoma was derived from I.29 cells taken from a different mouse at a different time (41). *SacI* produced a 6.1-kb fragment which contained both the expressed I.29 $C\alpha$ gene and the

FIG. 3. Southern blots of genomic DNAs from I.29 cells annealed with J_H probes. (A) Blots annealed with the J_{H3-4} probe. (B) Blots annealed with the J_{H1-2} probe. The sizes of the fragments indicated on the left side of each blot were determined relative to 32 P-labeled $HindIII$ fragments of λ bacteriophage DNA which were electrophoresed in a parallel lane in each blot. The restriction enzymes used are indicated below each blot.

$S\alpha$ sequences located 5' to the $C\alpha$ gene (Fig. 1A). If switch recombination occurred at sites separated from each other by ≥ 200 base pairs in different cells, we would easily have detected the difference in the sizes of the $C\alpha$ fragments. A total of about 30 different $SacI$ digestions which were electrophoresed on four DNA blots were compared, and no consistent difference in the sizes of the $SacI$ fragments containing the expressed α gene was detected (data not shown). Furthermore, the restriction fragments bearing J_H , $C\mu$, and $C\alpha$ genes in the noncloned population of FACS-sorted I.29 cells formed sharp bands on DNA blots that appeared to be as well defined as those obtained from the cloned BFO and hybridoma cells (Fig. 2; unpublished data). Thus, we found no evidence of heterogeneity among the sites of DNA recombinations in either the expressed or the nonexpressed H chain alleles in different I.29 cells.

Deletions of DNA sequences from the switch recombination sites. By comparing the map of the germ line $J_H-C\mu$ allele with those of the

expressed and nonexpressed $J_H-C\mu$ alleles in I.29 cells, it can be seen that DNA sequences were deleted from the intervening sequence between the J_H4 and the $C\mu$ genes (Fig. 1). The deletions occurred near the $S\mu$ sequences between the two $HindIII$ sites located approximately 1.5 and 5.3 kb 5' to the germ line $C\mu$ gene (Fig. 1). The expressed μ gene suffered a 0.5-kb deletion from the region in which switch recombination with the $C\alpha$ gene occurred in I.29 cells (Fig. 1A). Note that a $PstI$ site has also been lost from the region located 5' to the $S\mu$ sequences.

The two fragments from the nonexpressed allele which bore $C\mu$ underwent more extensive deletions than did those from the expressed allele in the $S\mu$ region and in the region just 5' to the $S\mu$ region. The larger fragment underwent a 1-kb deletion (Fig. 1B-1). The smaller fragment appeared to have undergone two additional DNA recombinations which resulted in the deletion of another 1 kb of nucleotides (Fig. 1B-2). The recombination event which entirely deleted the $C\mu$ gene from the nonexpressed chromo-

some also occurred within the $S\mu$ sequences and joined the $S\mu$ sequences with DNA sequences located 5' to the $C\gamma 3$ gene (Fig. 1B-3). The $S\mu$ region, in which the DNA recombinations occurred, consists of approximately 200 tandem repeats of a 10- to 40-base-pair sequence (26, 33; R. Greenberg and K. B. Marcu, manuscript in preparation). This region also undergoes deletions in myeloma DNAs and during the propagation of cloned μ genes in *E. coli* and is of variable length in the germ line DNA of different strains of mice (22, 23).

Structure of the $\gamma 2b$ genes in I.29 cells. If I.29 cells are actively switching and not simply related by previous switch recombination events, it is important to attempt to explain why I.29 cells switch only to the expression of IgA. The structure of the $\gamma 2b$ genes and the $S\gamma 2b$ region in I.29 cells was examined, using $\gamma 2b$ cDNA (36) and a plasmid pBR1.4 (18) which contained genomic DNA sequences from the S region located 5' to the $C\gamma 2b$ gene from BALB/c mice (Fig. 4A) to detect DNA fragments from I.29 cells produced by *Eco*RI, *Kpn*I, *Hind*III, *Bam*HI, or *Sac*I digestion. The results indicated that I.29 cells contain $\gamma 2b$ and $\gamma 2a$ genes in the germ line context (Fig. 4; unpublished data).

The intensity of the annealing with the $S\gamma 2b$ probe was greater in liver and in I.29 cells which express IgM (IgM.S) than in I.29 cells which express predominantly IgA (IgM.4P and IgM.5P) or only IgA (BFO) (Fig. 4B and C). This result is consistent with the presence of $\gamma 2b$ genes on both H chain chromosomes in IgM⁺ cells but on only one of the chromosomes in IgA⁺ cells. As an internal control for the amount of DNA loaded onto each lane, the annealing of the $S\gamma 2b$ probe with the blot containing *Sac*I fragments (Fig. 4B) was compared with the annealing of the same blot with the J_{H3-4} probe (Fig. 3A, *Sac*I). The intensity of the bands detected with the J_{H3-4} probe was approximately the same in the lanes containing DNA from liver, IgM.S, and BFO cells. Therefore, it appears likely that the switch from μ to α involves deletion of the $\gamma 2b$ gene from the expressed allele. These results suggested that there is no alteration or deletion of the $\gamma 2b$ genes or of the $S\gamma 2b$ sequences in I.29 cells, which would prevent switching from μ to $\gamma 2b$. In addition, the μ and α genes were not closely juxtaposed in IgM⁺ cells, as the restriction maps of the μ genes present in liver and in IgM⁺ cells were identical for a distance of at least 10.5 kb 3' to the $C\mu$ gene, and the maps of the α genes present in these same cells were identical for a distance of at least 9.5 kb 5' to the $C\alpha$ gene (Fig. 1A; unpublished data). Thus, the μ and α genes are separated by at least 20 kb in IgM⁺ cells.

Characteristics of the IgM.S cell line. An IgM⁺

FIG. 4. Restriction map of the $C\gamma 2b$ gene in germ line BALB/c cells and in I.29 cells. (A) This map (16, 18) corresponds with our map of the $C\gamma 2b$ gene in I.29 cells extending from the *Eco*RI site located 8 kb 5' to the $C\gamma 2b$ gene to the *Eco*RI site located 4 kb 3' to the $C\gamma 2b$ gene. The BALB/c DNA sequence contained within the $S\gamma 2b$ probe is indicated below the map. The location of the tandemly repeated sequences identified by Kataoka et al. (16) is indicated. The symbols for the enzyme sites are given in the legend to Fig. 1. (B) *Sac*I fragments annealed with the $S\gamma 2b$ probe. This blot is the same blot of *Sac*I fragments which is shown in Fig. 3A. The J_{H3-4} probe had been removed by heating, and the blot was subsequently annealed with the $S\gamma 2b$ probe. (C) Southern blot of I.29 DNAs digested with both *Bgl*II and *Pst*I and annealed with the $S\gamma 2b$ probe.

cell line (IgM.S) which grows only in vivo and does not switch to IgA expression was derived from the I.29 lymphoma by seeding approximately 10^3 IgM⁺ cells obtained by the FACS into each of several Millipore filter chambers which were then placed into the peritoneal cavity of (I/St $\times$ BALB/c) F_1 mice. Only one of the chambers yielded I.29 cells, all of which were IgM⁺. These IgM⁺ cells were then passaged intraperitoneally in mice for several passages, and only IgM⁺ cells were obtained. As was true for other IgM⁺ cells of the I.29 lymphoma, we

did not find conditions which would support the growth of the IgM.S cell line in vitro.

To attempt to determine whether IgM.S cells do not switch to IgA expression because of a deletion or other defect in their H chain genes, we compared the restriction enzyme maps of the H chain genes in the IgM.S cells with those of the H chain genes in the IgM hybridoma and mixed IgM⁺-IgA⁺ cell populations. No deletion or other defect in the H chain genes of the IgM.S cells was detected. The restriction maps of the expressed and nonexpressed *J_H-C μ* genes and of the α and $\gamma 2b$ genes in the IgM.S and IgM hybridoma cells were identical (excluding the P3-NS.1-Ag-8 contribution to the hybridoma cell line [Fig. 1 through 4; unpublished data]). In both the IgM.S and IgM hybridoma cells, there was only one *Kpn* fragment from the nonexpressed μ allele, the intermediate-sized (10-kb) fragment. Apparently, the IgM.S and IgM hybridoma cells were both arrested at the same stage of, or before, the switching process. We do not know the frequency of occurrence of cells such as the IgM.S cells in the I.29 lymphoma, although by examining the μ genes in IgM.1P cells (Fig. 2A), we can conclude that no more than 50% of the IgM.1P cells have the nonexpressed μ gene in the same form as that found in IgM.S cells.

Rearrangement of light chain genes in I.29 cells. I.29 cells express a λ light chain. Cell lines which express λ chains usually have rearranged or deleted both of their *C κ* genes, in addition to having rearranged one or more *C λ* genes. By contrast, cells which synthesize κ chains do not contain rearranged λ genes (6, 11). Our data are consistent with the results in other systems. When *EcoRI* or *BamHI* fragments of IgM.1P and BFO cell DNAs were annealed with a *C κ* cDNA probe (pM384 κ [40]), one rearranged *C κ* fragment of identical size was detected in both of these types of cells (Fig. 5A), whereas annealing a probe for a $\lambda 1$ light chain (2) with *EcoRI* or *BamHI* digests of IgM.1P and BFO cell DNAs revealed two rearranged λ fragments in addition to the germ line λ fragments (Fig. 5B). These results provide further proof that the IgM⁺ and IgA⁺ cells of the I.29 lymphoma were derived from the same parental cell.

DISCUSSION

Are I.29 cells undergoing active switching? The I.29 lymphoma may contain cells which can switch from the synthesis of immunoglobulin μ chains to the synthesis of immunoglobulin α chains. However, since IgM⁺ cells have not been cloned from the lymphoma, one could alternatively hypothesize that the I.29 lymphoma consists of four distinct types of cells which are related by previous switch events and which

FIG. 5. Southern blots of genomic DNAs annealed with probes for immunoglobulin light chain genes. (A) Blot annealed with *C κ* cDNA. (B) Blot annealed with λ H2020 cDNA. The positions of the *HindIII* fragments of λ DNA which were electrophoresed in each gel are indicated on the left. The enzymes used are indicated at the bottom of the lanes.

have continued to coexist in the transplanted tumor during 21 years of in vivo passage. Our Southern blotting experiments indicated that there are two different types of IgM⁺ cells, those containing the nonexpressed μ allele in the form of the 11-kb *Kpn* fragment and those containing the nonexpressed μ allele in the form of the 10-kb *Kpn* fragment. Karyotype analyses indicated that the I.29 cells are diploid; thus, these two different forms of the nonexpressed allele do not exist in the same cell. Furthermore, cloned IgM⁺ hybridoma cells (ID43) and the IgM⁺ line which has never been observed to switch (IgM.S) contain only one of the forms of the nonexpressed allele (the 10-kb *Kpn* fragment). The other cell types present in the lymphoma are the IgA⁺ cells and the double-producing cells. The presence of double-producing cells constitutes the strongest argument for the occurrence of active switching, as double-producing cells could be cells which have recently switched to synthesis of α chains but still contain residual μ mRNA and μ chains.

If I.29 cells are undergoing active switching, it is necessary to explain why the tumor does not become 100% IgA⁺; the IgA⁺ cells cannot revert to IgM expression, since our data proved that the μ genes have been deleted from IgA⁺ cells. One could hypothesize that switching occurs during DNA replication (43), that only one of the two daughter cells switches to IgA⁺, and that, thus, the IgM⁺ cells would not be depleted. The presence of IgM.S-like cells in the lymphoma cannot explain why the tumor never be-

comes 100% IgA⁺, because if so, the tumor should eventually consist of two separate populations, IgM.S and IgA⁺ cells.

Rearrangement at the nonexpressed *J_H* locus may involve *D-J* joining without *V-D* joining. The structures of the DNA rearrangements in three examples of aberrantly rearranged *J_H* genes found in B and T cells have been determined (17, 32). In all three examples, a *D* gene was joined to the *J_{H2}* gene without association with a *V_H* gene; in two of these cases, the identical *D* gene (*D_{Q52}*), which is located 0.7 kb 5' to *J_{H1}* in germ line DNA, was joined to the *J_{H2}* gene. In I.29 cells, the same recombination of the *D_{Q52}* gene with *J_{H2}* may have occurred at the nonexpressed *J-C_μ* allele, as the restriction map of the region 5' to the *J_{H2}* gene was similar to the map of the *D_{Q52}-J_{H2}* rearrangement. The map of the *D_{Q52}-J_{H2}* rearrangement is shown below Fig. 1B-3 (32). The mechanism of allelic exclusion in I.29 cells could involve the recombination of a *D* gene with a *J* gene without association with a *V_H* gene.

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