LAH 276

Homologous chromosome recombination generating immuno-globulin allotype and isotype switch variants

T.J.Kipps[1] and L.A.Herzenberg

Department of Genetics, Stanford University, Stanford, CA 94305, USA

[1]Present address: Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, 10666 N. Torrey Pines Rd., La Jolla, CA 92037, USA

Communicated by K.Rajewsky

We investigated whether spontaneous isotype switching in monoclonal antibody-producing hybridomas always occurs with genes on the same chromosome. Spleen cells of (BAB/25 × AKR/J) F1 mice, immunized with dansyl-keyhole limpet hemocyanin (DNS-KLH), were hybridized with NS-1 to generate hybridomas producing monoclonal anti-DNS antibodies of either the b or d haplotype of the BAB/25 or AKR/J parent, respectively. We selected isotype switch variants of such hybridomas using the fluorescence-activated cell sorter (FACS). Although in most cases the allotypic haplotype expressed by the parent and switch-variant hybridomas are the same, in one family of variants we noted a switch in haplotype along with the switch in isotype. This was noted in the selection of IgG_{2a} switch variants from an IgG_1 switch variant originally derived from an IgG_3-producing parent. Biochemical and molecular studies confirm that the allotype switch variant expresses the same heavy-chain variable region gene complex as its parent hybridomas. As such, the allotype switch represents an example of spontaneous mitotic recombination between immunoglobulin heavy-chain genes, generating a single actively transcribed gene from loci previously positioned on different chromosomes.

Key words: isotype switching/hybridomas/monoclonal antibodies/chromosome recombination/allotype

Introduction

Immunoglobulin (Ig) heavy-chain class switching is a process of normal B-cell differentiation that occurs spontaneously in cultured murine myelomas and monoclonal antibody-producing hybridomas at frequencies ranging from 10^{-4} to 10^{-7}/cell/generation (Preud'homme et al., 1975; Liesegang et al., 1978; Radbruch et al., 1980; Neuberger and Rajewsky, 1981; Beyreuther et al., 1981; Dangl et al., 1982; Kipps and Herzenberg, 1984). Using the fluorescence-activated cell sorter (FACS), isotype switch variants can be isolated by sorting for cells expressing variant surface immunoglobulin, allowing one to study immunoglobulin gene expression in both parent and switch-variant progeny (Liesegang et al., 1978). In vitro isotype switching in cultured cells apparently results from DNA rearrangement(s) in the heavy-chain immunoglobulin gene complex similar to those found in plasmacytomas. The newly expressed heavy-chain gene is found 3' of the expressed VDJ gene, the exons coding for the formerly expressed isotype having been deleted (Sablitzky et al., 1982). Although the sites at which such rearrangement(s) occur are found outside the physiologic switch regions (S), isotype switch variants generally produce antibodies that are the same

as physiologically switched antibodies of the same isotype in mol. wt. glycosylation and primary protein sequence.

One unusual aspect of in vitro isotype switching is that some isotype switch variant hybridomas are noted to revert to expressing the original parental isotype at frequencies comparable with or greater than that of the original 'forward' switch (Beyreuther et al., 1981; Dangl et al., 1982). Because upstream isotype genes are often deleted in switch variant hybridomas, such 'backswitching' is difficult to explain. One hypothesis proposes that unequal mitotic recombination between the immunoglobulin isotype genes on homologous chromosomes may permit the re-expression of an upstream antibody isotype gene previously deleted from the expressed heavy-chain immunoglobulin gene complex. Some evidence for this has been obtained from restriction enzyme analyses of genomic DNA of these hybridoma revertants (Sablitzky et al., 1982).

To investigate whether antibody isotype switching in hybridomas may involve mitotic recombination between the immunoglobulin genes, we constructed murine hybridomas from an F1 animal derived from parents with different immunoglobulin haplotypes (Herzenberg and Warner, 1967; Herzenberg et al., 1968; Herzenberg and Herzenberg, 1978). In so doing, we could monitor the expression of the immunoglobulin alleles by distinguishing the allotype of the expressed antibody using several well-characterized murine monoclonal anti-allotypic antibodies (Oi and Herzenberg, 1979; Parsons et al., 1981; Oi et al., 1983; Huang et al., 1983). This report describes our experience in selecting immunoglobulin isotype switch variants from such hybridomas.

Results

Isolation of the 2.30 switch variant family

Using FACS we selected a large family of switch variant hybridomas from an IgG_3 anti-dansyl (DNS) antibody-producing hybridoma, 2.30 (Figure 1). Most variants were identified by staining cells with flurochrome-conjugated heterologous antibodies specific for the variant isotype. During the first rounds of FACS sorting, we selected the brightest 0.1% of $1-2 \times 10^7$ labeled hybridoma cells. These cells were then grown to $>5 \times 10^6$ cells, relabeled and sorted again for the variant isotype.

After sufficient enrichment, single cells were cloned into individual wells of a 96-well microtiter plate. Identified switch variant clones were cultured for additional variant selections. The variant V107 was isolated from the original parental 2.30 population using fluorescein isothiocyanate (FITC)-goat anti-mouse IgG_1. The IgG_{2a} and IgG_{2b} variants, V19 and V14, respectively, were derived from the cloned V107 variant by staining with FITC-goat anti-mouse IgG_2. Another IgG_{2a} variant, V17, was derived from V14 by selecting for variants staining with FITC-goat anti-mouse IgG_{2a}. The IgE and IgA variants, V19-7.1 and V19-2.2, respectively, were selected from V19 using two-color immunofluoresence, sorting for hybridomas that had lost surface IgG_{2a} expression but retained DNS binding activity. Subsequently,
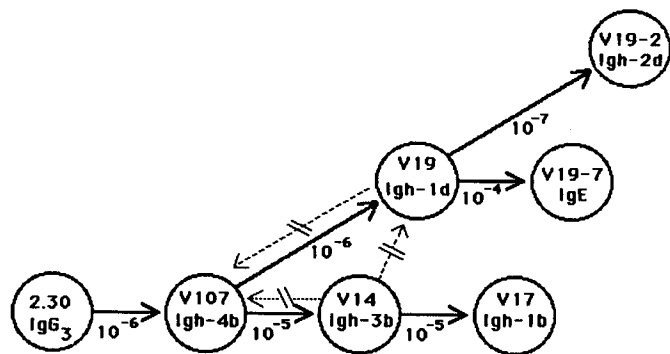
Fig. 1. 2.30 Switch-variant family. Solid arrows represent successful switch-variant selections. The approximate proportions of variants noted with each selection are listed below the arrows. Broken arrows represent unsuccessful selections.

Table I. Reactivity of the anti-DNS antibodies with monoclonal anti-allotype antibodies

Anti-allotype	Specificity	Anti-DNS monoclonal antibodies					
		2.30 (IgG$_3$)	V107 (IgG$_1$)	V14 (IgG$_{2b}$)	V17 (IgG$_{2a}$)	V19 (IgG$_{2a}$)	V19-2.2 (IgA)
412-79.2	Igh-4[b]	−	+	−	−	−	−
9.10	Igh-4[a/d]	−	−	−	−	−	−
417-72.1	Igh-3[b]	−	−	+	−	−	−
9.8	Igh-1[a/d]	−	−	−	−	+	−
8.3	Igh-1[a/d]	−	−	−	−	+	−
16.3	Igh-1.3[a/d]	−	−	−	−	+	−
BV-46	Igh-1[d]	N.T.[b]	N.T.	−	−	+	−
5.7	Igh-1[b]	−	−	−	+	−	−
2.9	Igh-1[b]	−	−	−	+	−	N.T.
UC-1	Igh-2[a]	N.T.	N.T.	N.T.	−	−	−
HY-16	Igh-2[a/d]	N.T.	N.T.	−	−	−	+

[a]Antibody heavy-chain isotype corresponding to each immunoglobulin locus: Igh-1 (IgG$_{2a}$), Igh-2 (IgA), Igh-3 (IgG$_{2b}$) and Igh-4 (IgG$_1$).
[b]N.T. = not tested.

FITC-labeled isotype-specific goat antibodies were used to clone variants from antigen-binding hybridomas that had lost surface IgG$_{2a}$ expression. The frequencies listed in Figure 1 are approximations derived from the product of sort frequencies of successive selections multiplied by the proportion of isotype variants identified among clones isolated in the final sort. As indicated by the broken arrows in Figure 1, we were not successful in selecting 'backswitch' variants of the IgG$_2$ hybridomas despite several successive rounds of sorting for IgG$_1$-expressing variants of V19 or V14 hybridomas.

Allotype of selected isotype switch variants

We determined the allotype of selected hybridomas using a solid-phase radioimmunoassay. Anti-DNS antibodies binding DNS$_{20}$-bovine serum albumin (BSA) coated polystyrene microtiter plates were detected with radiolabeled murine monoclonal anti-allotype antibodies (Table I). As the IgG$_3$ isotype has no known polymorphisms in inbred strains of mice, we cannot determine the allotype of the parent hybridoma (Huang *et al.*, 1982). The IgG$_1$ switch variant, V107, produces anti-DNS antibodies of the b haplotype, specifically binding 412-79.2 anti-Igh-4b. However, the IgG$_2$ switch variant progeny, V19 and V14, are of the d haplotype and b haplotype, respectively. V19 was initially identified using anti-allotypic antibodies directed against the Igh-1a allotype with which the Igh-1d allotype of AKR/J mice shares many cross-reactive specificities (Huang *et al.*, 1983), (designated in this paper as 'a/d' determinants). However, the IgG$_{2a}$ antibody produced by V19 is of the AKR/J d haplotype, and not of the a haplotype of the NS-1 myeloma fusion partner, as it is recognized by BV-46, a BALB/c-derived Igh-1a monoclonal antibody specific for Igh-1d determinants. V17, the IgG$_{2a}$ switch variant of V14, retained expression of the b haplotype, as recognised by Igh-1b specific monoclonal antibodies 5.7 and 2.9. The IgA variant, V19-2.2, derived from V19, expresses antibodies recognized by HY16, an Igh-3e anti Igh-2a/d monoclonal antibody. These IgA antibodies, however, failed to react with AKR/J-derived monoclonal antibodies, UC-1, specific for Igh-2a. All anti-DNS antibodies tested were reactive with 187-1, a rat monoclonal antibody specific for mouse kappa light chain.

The allotypic determinants recognized by several of these monoclonal antibodies have been mapped to specific immunoglobulin domains, allowing us to confirm the allotypic haplotype of the expressed anti-DNS antibodies at different sites along the immunoglobulin molecule (Oi and Herzenberg, 1979; Parsons *et al.*, 1983; Oi *et al.*, 1983). The anti-DNS antibodies of V19, for example, react with non-cross-blocking antibodies 9.8 and

8.3, these recognizing Igh-1a/d determinants mapped to the CH2 and CH3 domains of IgG$_{2a}$, respectively. Moreover, V17 anti-DNS antibodies are recognized by 2.9 and 5.7, anti-1b antibodies specific for sites mapped to CH2 or CH3, respectively.

*Biochemical evaluation of switch variant antibodies*

The antibodies produced by individual members of this switch variant family were analyzed via two-dimensional gel electrophoresis. The antibody heavy chains of each are ~53−55 kd, the mol. wt. of intact mouse immunoglobulin gamma heavy chain (Figure 2). Furthermore, the V14 heavy chain has a double-band pattern common to glycosylated murine IgG$_{2b}$ heavy chains. In contrast to the heavy chains which have different pI values, all light chains have the same pI of ~4.8.

Purified switch variant antibodies were tested for binding activity with free ε-dansyl-L-lysine. In contrast to previously studied monoclonal anti-DNS antibodies of high affinity (Oi *et al.*, 1984), the antibodies of this family only marginally enhanced the fluorescence of dansyl-lysine. However, anti-DNS antibody from each member of this switch variant family produced the same characteristic blue shift in the fluorescence of dansyl-lysine, consistent with all antibodies having the same binding activity for ε-dansyl-L-lysine (not shown).

Molecular characterization of switch variant hybridomas

Restriction enzyme analysis of genomic DNA reveals that all members of this switch variant family share a common immunoglobulin VDJ rearrangement. Southern blot analyses of *Eco*RI-digested DNA probed with nick-translated ^{32}P-labeled pJ$_H$3,4, for example, revealed each hybridoma to have a rearranged band of 3.2 kb in addition to the 6.6-kb band of the NS-1 fusion partner (Figure 3). All hybridomas, with the exception of V19 and its subsequent switch variant progeny, have an additional rearranged band of 4.5 kb. Southern analyses of *Hind*III-digested DNA also demonstrated all hybridomas to share the 2.2-kb band of the NS-1 fusion partner as well as a rearranged band of 4.3 kb (not shown). Another rearranged band of 12.5 kb is common only to the IgG$_3$ parent 2.30 and switch variant progeny of the b haplotype.

To verify that the same heavy-chain VDJ is expressed by different hybridomas of this switch variant family, we analyzed the sequence of the expressed antibody heavy-chain mRNA. Using synthetic oligonucleotide 15-mers or 14-mers of conserved se-
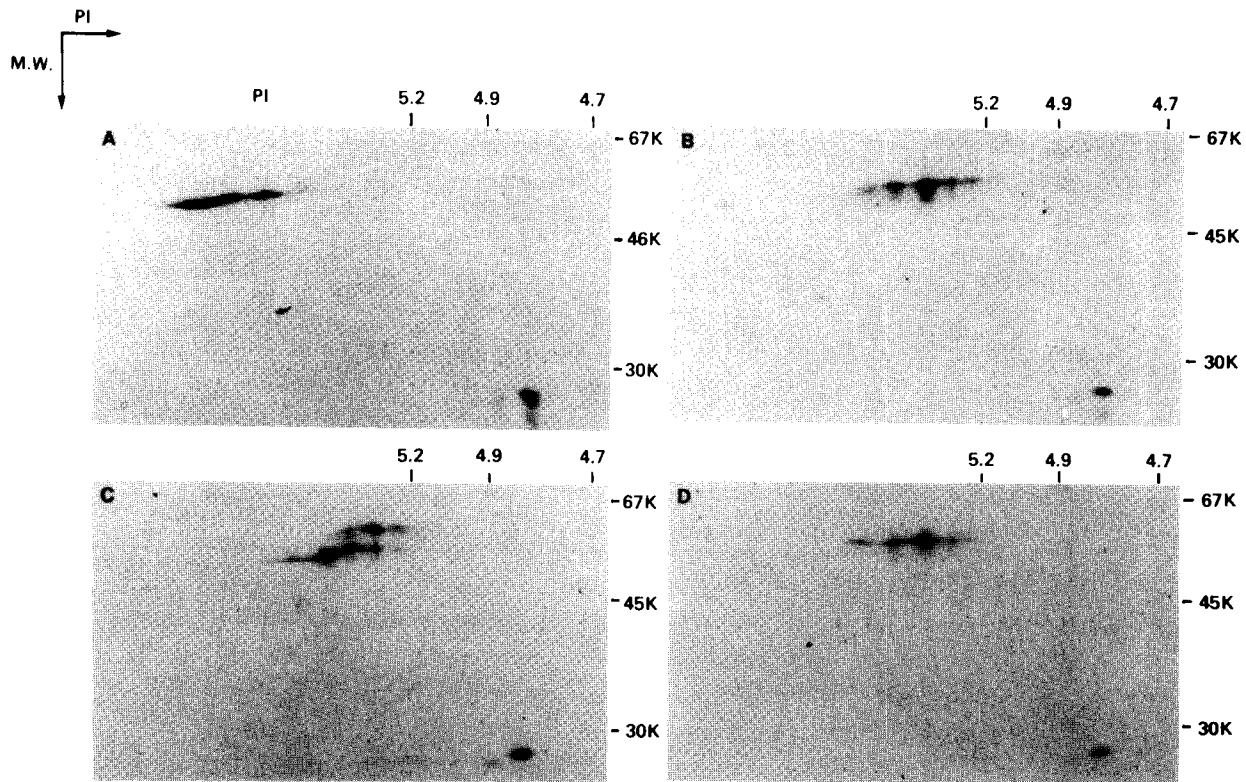
Fig. 2. Two-dimensional gel electrophoresis of the secreted monoclonal antibodies produced by various members of the 2.30 switch-variant family. Reduced and biosynthetically ^{35}S-labeled anti-DNS antibodies were separated in the first dimension via non-equilibrium pH gradient electrophoresis and in the second dimension via SDS-polyacrylamide gel electrophoresis. Listed are pI values and mol. wts of added protein standards (Pharmacia). Samples are: (A) 2.30; (B) V107; (C) V14; and (D) V19.

quences in C_H or J_H, respectively, we specifically primed synthesis of cDNA on purified poly(A)-rich mRNA. The variable region sequences of the parent hybridoma, 2.30, and switch variant progeny V107, V14 and V19 were determined from J to the second hypervariable region (Figure 4). All hybridomas express the same V_HIIb, D segment and J_H4, thus confirming the clonal origin of this switch variant family.

Discussion

To investigate whether antibody isotype switching in hybridomas may involve mitotic recombination between the immunoglobulin genes on homologous chromosomes, we constructed murine hybridomas from an F1 animal derived from parents with different immunoglobulin haplotypes. This allowed us to monitor the expression of the immunoglobulin alleles by distinguishing the allotype of the expressed antibody using several well-characterized murine monoclonal anti-allotypic antibodies. We selected isotype switch variants of such hybridomas using the FACS. Although in most cases the allotypic haplotypes expressed by the parent and switch variant hybridomas are the same, in one family of variants we noted a change to an allotype included in the other parental haplotype along with the switch in isotype. This was noted in the selection of IgG_{2a} switch variants from an IgG_1 switch variant originally derived from an IgG_3-producing parent. Biochemical and molecular studies confirm that the allotype switch variant expressed the same heavy-chain variable-region gene complex as its parental hybridoma. As such, the allotype switch represents an example of spontaneous mitotic recombination between immunoglobulin heavy-chain genes, generating
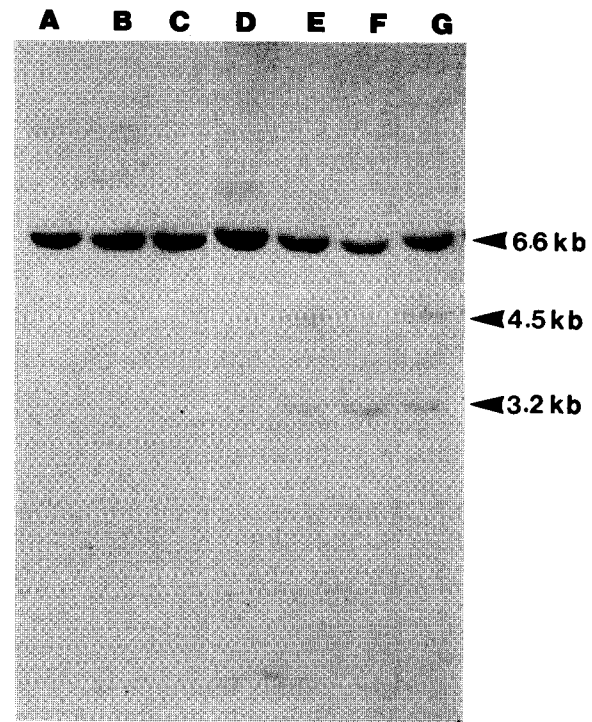
Fig. 3. Autoradiogram of Southern blot analysis of EcoRI-digested genomic DNA probed with pJ_H34. Arrows indicate the kilobases of detectable bands. Sample lanes represent analyses of genomic DNA extracted from: (A) AKR/J liver; (B) BAB/25 liver; (C) (AKR/J × BAB/25) F1 liver; (D) NS-1; (E) 2.30; (F) V19; (G) V14.

CDR2

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                              60                65        70
              X   X   X   T   R   T   T   Q   K   F   K   G   K   A   T   L   T   A   D   K   S
V107 (Igh-4b) A  XAT GXX XAT ACT AGG TAC ACT CAG AAG TTC AAG GGC AAG GCC ACA TTG ACT GCA GAT AAA TCC

V19  (Igh-1d) -- --- --- X-- --- --X- --- --- --- --- X-- --- --- --- --- --- --- --- --- XXX XXX

V14  (Igh-3b)    --- --- --- --- --- --- --- X-- --- --- --- --- --- --- --- --- -X- ---

2.30 (IgG3)                                  --- --- --- --- --- XXX ---
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              75              80         a   b   c       85              90
              S   S   T   A   S   M   Q   L   S   S   L   A   S   E   D   S   G   X   Y   Y   C   G
V107 (Igh-4b) TCC AGC ACA GCC TAC ATG CAA CTG AGC AGC TTG GCA TCT GAG GAC TCT GGX GXC TAT TAC TGT GGA

V19  (Igh-1d) X-- --- X-- --- --- --- --- --- --- --- X-- --- --- --- --- ---G --- --- --- --- --X

V14  (Igh-3b) --- --- --- --- --- --- --- --- --- --- ---- --- --- --- ---G --- --- --- --- ---

2.30 (IgG3)   --- --- X-- --- --- --- --- -X- --- --- --- --- --- -XX --- --G --- --- --- --- --X
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D   **J_H**

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              |95           ||100   100C         105            110
              R   C   L   P   Y   A   Y   Y   A   M   D   Y   W   G   Q   G   T   S   X   T   V   S   S
V107 (Igh-4b) AGA TGT CTX CCC TAT GCA TAC TAT GCT ATG GAC TAC TGG GGT XXX GGA ACC TCA GTX ACC GTC TCC TCA

V19  (Igh-1d) -- --- --- --- --- --- --- --- --- --- --- --- --- CAA --- X-- --X

V14  (Igh-3b) -- --- --- --- --- --X XX- --- --- --- --- --- --- -AA --- ---

2.30 (IgG3)   -- --- --- --- --- --X --- --- --- --- --- --- --- ---

J_H4                                              --- --- --- --- --- --- --- --- CAA --- --- --- --C --- --- --- ---
```
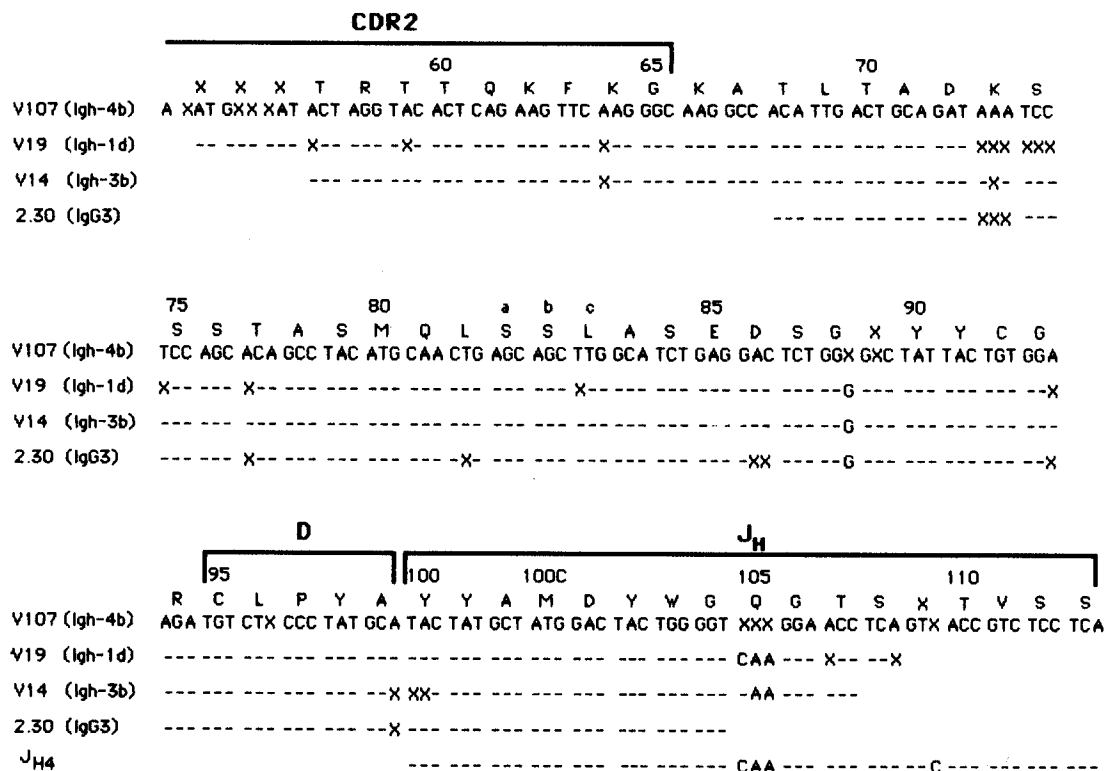
Fig. 4. The nucleotide sequence obtained via oligonucleotide-primer extension of extracted poly(A)[+] RNA. Ambiguities of the mRNA sequences are indicated by X. The sequence of J_H4 (Sakano *et al.*, 1980) is listed for comparison. Numbering is according to Kabat (Kabat *et al.*, 1983). Listed above each codon is the deduced amino acid sequence: 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.

a single actively transcribed gene from loci previously positioned on different chromosomes.

The frequency of allotypic haplotype exchange is low compared with the switching of isotypes located on the same chromosome. In this regard, the isolation of the original V19 allotype variant may have been quite fortuitous. Using similar selection techniques to those employed in obtaining this Igh-1d hybridoma, we isolated only Igh-3- and Igh-1b-expressing switch variant hybridomas from four different subclones of the parent Igh-4b-expressing hybridoma, V107 (not shown). Moreover, only Igh-1b variants were isolated from the Igh-3b-producing hybridoma, V14. Attempts to select Igh-1d-expressing variants from V14 or V107 subclones with FITC-labeled monoclonal anti-Igh-1a (8.3) were equally frustrating (not shown). Furthermore, after having exchanged immunoglobulin haplotypes, the Igh-1d variant, V19, generated subsequent IgA and IgE isotype switch variants expressing allotypic determinants of the same d haplotype.

The frequency of 'backswitches' to isotypes 5' or upstream in this family was below detectability ($\sim 10^{-7}$). Several attempts at selecting IgG_1-expressing variants from either V19 or V14 were unsuccessful. Moreover, selection for variants of V19 that expressed surface immunoglobulin which retained antigen-binding capacity without IgG_{2a} determinants selected only 'forward' 3' or downstream switch variants expressing IgE and IgA. Perhaps the low rate of mitotic cross-over is responsible for our failure to obtain more than one allotype exchange and any 'backswitch' IgG_1 expressing variants of the IgG_2-producing hybridomas. Alternatively, the IgG_2 switch variants may have deleted the functional exons encoding the IgG_1 heavy-chain isotype during the process of isotype switching.

Because of the low frequency of allotypic exchange, it became

particularly important to determine the VDJ of the switch variants in this family. All the variants of this family produced antibodies that bind dansylated BSA and have similar association constants for free dansyl-L-lysine. Furthermore, these anti-DNS antibodies have kappa chains of identical pI, consistent with the conserved expression of the same light chain by all members of this hybridoma family. Restriction enzyme analyses of genomic DNA revealed all members of the family share a common heavy-chain VDJ rearrangement. That this VDJ complex in fact encodes the variable region of the expressed heavy chain was determined definitively by sequencing the variable region of the immunoglobulin mRNA of 2.30, V107, V14 and V19. These data provide conclusive evidence for the clonal origin of this hybridoma family, and also rule out the unlikely possibility that another variable region complex on the formerly non-expressed chromosome is activated in the allotype switch variants.

Unequal and/or non-reciprocal recombination between the immunoglobulin isotype genes may be the mechanism of immunoglobulin isotype switching. It has been proposed that such recombination may generally occur between immunoglobulin isotype genes present on sister chromatids (Honjo and Kataoka, 1978; Shimizu and Honjo, 1984). Recently, however, this model has been challenged (Wabl *et al.*, 1985). Wabl and his colleagues analyzed the arrangement of C-mu DNA in many different clones of the pre-B-cell line 18-81 that switches *in vitro* from IgM to IgG_{2b} (Burrows *et al.*, 1983; Alt *et al.*, 1982; Wabl *et al.*, 1984). In none of these clones could the hypothesized reciprocal genetic material of a recombinatorial event be found. Their findings exclude unequal sister chromatid recombination as the mechanism for the isotype switch observed in this cell line, unless such exchange is non-reciprocal. It is perhaps relevant that the isotype

switch from Igh-4b to Igh-1d is associated with the loss of a re-arranged J segment. This loss may be secondary to non-reciprocal recombination between the immunoglobulin isotype genes with subsequent loss of the abortive variable region rearrangement on the formerly non-expressed chromosome. Rather than non-reciprocal recombination between sister chromatids, the allotype switch may represent a rather unusual pairing and subsequent non-reciprocal exchange between the immunoglobulin heavy-chain genes of different homologous chromosomes, resulting in mitotic recombination.

Spontaneous mitotic recombination has been demonstrated in several eucaryotic organisms (Stern, 1936; Pontecorvo and Kafer, 1958; Vig and Paddock, 1968; Katz and Kao, 1974) and recently in somatic mammalian cells grown in culture (Wasmuth and Hall, 1984). Mitotic recombination has been implicated in isotype 'backswitching' of cultured switch variant hybridomas (Sablitzky et al., 1982), somatic mutation of heavy-chain variable region genes (Dildrop et al., 1982; Clarke and Rudikoff, 1984), and in the generation of hybrid gamma2b-gamma2a heavy-chain pro-ducing variants from MPC-11 after mutagenesis with ICR 11.19.3 (Tilley et al., 1983).

Recombination between highly homologous genes within multi-gene families may be an important mechanism at the meiotic level for generating allotypic polymorphisms in both constant region and variable region genes (Herzenberg et al., 1968; Gally and Edelman, 1970; Capra and Kindt, 1975; Baltimore, 1981; Ollo and Rougeon, 1982, 1983; Kvist et al., 1983). The capacity to study such recombination at the somatic level in cultured hybrid-omas may shed light on the in vivo mechanisms of these re-arrangements.

Materials and methods

Animals

Mice were raised and maintained in the Herzenberg breeding colony.

Antibodies

BV46 was obtained from Dean Ballard, University of Illinois, Urbana. HY16, developed by M.Potter, was obtained from C.Muller, Stanford University. All other monoclonal antiallotype antibodies (Table I) were generated in our laboratory (Oi and Herzenberg, 1979; Parsons et al., 1981; Huang et al., 1983). Rat anti-mouse kappa, 187.1, developed by D.Yelton and M.D.Scharff (Yelton et al., 1981) was obtained from the American Type Culture Collection (Rockville, MD). Heterologous goat antibodies specific for mouse immunoglobulin isotypes were prepared by solid-state absorption and elution (e.g. Parham et al., 1983).

Hybridomas

Cells were cultured in RPMI-1640 (GIBCO) supplemented with 2 mM L-glutamine and 10% horse serum at 37°C in a 7% CO_2-in-air incubator. The parent hybrid-oma, 2.30, was isolated from the fusion of NS-1 with spleen cells of (AKR/J × BAB/25) F1 mice primed and boosted with dansyl-keyhole limpet hemocyanin. Hybridization was performed with polyethylene glycol and subsequent selection in culture medium containing hypoxanthine, aminopterin and thymidine (Oi and Herzenberg, 1980).

Dansylation

Protein was solubilized in 0.5 M $NaHCO_3$ (pH 9.5) at concentrations ranging from 0.5 to 1.5 × 10^{-4} M. 5-Dimethylaminonaphthalene-1-sulfonyl chloride (dansyl-chloride, Sigma, MO) was dissolved in dimethylformamide to 10 mg/ml. A 25 M excess of dansyl chloride was added slowly to a rapidly stirring solution of protein and allowed to react at 4°C overnight. Unreacted dansyl chloride was separated from dansylated protein by G25-Sephadex chromatography. The approximate numbers of dansyl molecules bound per molecule of protein was determined spectrophotometrically at 280 and 340 nm.

Fluorescence-activated cell sorting

One- or two-color fluorescence-activated cell sorting was performed as describ-ed (Parks and Herzenberg, 1984), on a modified FACS II equipped with an argon laser and a variable-frequency adjustable rhodamine-dye laser. Sorting for hybrid-omas expressing anti-DNS surface immunoglobulin was achieved by staining cells with a phycobiliprotein, allophycocyanin (Oi et al., 1982) obtained from Dr

D.R.Parks, Stanford University, that had been coupled to dansyl-chloride. Allo-phycocyanin excitation at 600 nm was monitored at 640 nm.

Two-dimensional gel electrophoresis

For biosynthetic labeling, 5 × 10^7 hybridoma cells were cultured for 5 h in 2 ml methionine-free culture medium supplemented with 5% fetal calf serum (FCS) and 0.5 mCi [^{35}S]methionine, 1000 Ci/mmol (New England Nuclear). Harvested supernates were added to polystyrene plates previously coated with DNS_{20}-BSA. After 1 h of incubation at room temperature, plates were extensively washed with phosphate-buffered saline (PBS) supplemented with 1% BSA. Specifically bound anti-DNS antibody was washed off the plate using Nonidet P-40 sample buffer for two-dimensional gel electrophoresis, as described (Jones, 1980).

Spectroscopic measurements

Fluorescence spectra were obtained on a Perkin-Elmer model 44B fluorimeter equipped with a DCSCU-2 corrected emission spectra unit. The fluorescence of purified murine monoclonal antibodies with ε-dansyl-L-lysine (Sigma, MO) were compared with the fluorescence of ε-dansyl-L-lysine with non-specific purified chicken gamma globulin (Sigma, MO), at antibody concentrations ranging from 5 × 10^{-7} to 10^{-5} M in 0.15 M NaCl, 0.05 M Tris pH 8.2.

Radioimmune assays

Polystyrene microtiter plates were coated with DNS_{20}-BSA at 50 µg/ml for 1 h. Radioimmune assays (RIA) were performed as described (Tsu and Herzenberg, 1980).

Analysis of genomic DNA

Genomic DNA of freshly isolated hepatocytes and cell lines were isolated as de-scribed (Blin and Stafford, 1976). 10 µg samples of DNA were digested with 50 U of EcoRI or HindIII (New England Biolabs) size separated on gels of 0.8% agarose (100 mA, 40 V for 12 h) and transferred onto nitrocellulose (Southern, 1975). Hybridization was performed as described (Migone et al., 1983), using pJ34 labeled with ^{32}P via nick translation to a specific activity of $1-3 × 10^8$ d.p.m./µg (Rigby et al., 1977). pJ_H34, a 1.6-kb genomic EcoRI-BamHI frag-ment spanning the third and fourth J_H segments subcloned into pBR322 (Sakano et al., 1980), was obtained from J.Dangl, Stanford University.

RNA sequencing

RNA was extracted from each of the cell lines with 6 M guanidium isothio-cyanate followed by CsCl density-gradient centrifugation (Glisin et al., 1974). Poly(A)$^+$ RNA was purified using oligo(dT)-cellulose (Edmonds et al., 1971). Synthetic oligonucleotide DNA primers were used to initiate cDNA synthesis by reverse transcriptase on poly(A)$^+$ RNA. Specific priming of cDNA synthesis was accomplished using either a 15-bp oligonucleotide C-gamma primer (3'-CAGATAGGTGACCGG-5') or a mixture of two 14-bp primers (J1 primer: 3'-CAGACCCCGTGTCC-5'; J2-4 primer: 3'-ATGACCCCGGTTCC-5'), as described (Kaartinen et al., 1983; Sablitzky and Rajewsky, 1984). These primers were provided by Dr Fred Sablitzky (Universitat zu Köln, Köln, FRG). The cDNA synthesis with either [^{32}P]ATP or [^{32}P]CTP was specifically terminated using dideoxynucleotides to determine the RNA sequence (Hamlyn et al., 1978, 1981).

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