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Development of the Primary Antibody Repertoire

FREDERICK W. ALT, T. KEITH BLACKWELL, GEORGE D. YANCOPOULOS

The ability to generate a diverse immune response depends on the somatic assembly of genes that encode the antigen-binding portions of immunoglobulin molecules. In this article, we discuss the mechanism and control of these genomic rearrangement events and how aspects of this process are involved in generating the primary antibody repertoire.

HUMORAL IMMUNITY IN VERTEBRATES IS EFFECTED BY antigen-binding antibody [or immunoglobulin (Ig)] molecules that are secreted by cells of the B lymphocyte lineage. The differentiation of B-lineage cells can be divided into two general stages (*1*) (Fig. 1). The first stage is antigen-independent and involves differentiation of stem cells to B lymphocytes, cells defined by the presence of surface Ig that functions as an antigen receptor. In mammals, this process occurs in the liver of the fetus and subsequently is maintained continuously in the bone marrow of adults. Each newly generated B cell (and its progeny) expresses a novel species of Ig molecule on its surface with a unique set of binding specificities. After acquiring antigen receptors, B lymphocytes migrate to peripheral lymphoid organs, such as the spleen and lymph nodes, where contact occurs between lymphocytes and circulating antigens (*1*). In the absence of further stimulation, these peripheral B lymphocytes are nondividing (resting) cells. The second stage of B cell differentiation (antigen-dependent) involves specific binding of antigen to the surface Ig receptor of a resting B cell. This process induces that cell to proliferate; its progeny can then differentiate into plasma cells, the effector cells of the humoral immune system, which secrete into the bloodstream large amounts of an Ig molecule with the same binding specificity as that present on the membrane of the progenitor B cell (*2*).

The membrane-bound form of an Ig molecule consists of two identical heavy and two identical light Ig chains (HC and LC, respectively) (Fig. 2). The carboxyl termini of H and L chains have a constant amino acid sequence (C region) among antibodies of the same class. The amino termini of both HCs and LCs contain regions that vary in amino acid sequence among different clonally derived sets of B lymphocytes; these variable regions are encoded by genes which are assembled from component segments (*3*) in precursor B (pre-B) cells during the antigen-independent stages of B cell development [reviewed in (*4*)]. The variable regions of H and L chains interact to form the antigen-binding site, which determines the particular binding specificities of the Ig molecule. Somatic assembly of variable region genes, together with the combinatorial assortment of different H and L chains, frees the immune system from the limitations of a germline-encoded set of responses and provides the animal with the potential to create an almost infinite number of different antigen-binding specificities. However, the specificity of the immune response depends on antigenic selection of discrete clones of B cells, each restricted to expression of a homogeneous set of Ig receptors [clonal selection; Fig. 1 (*2*)]. Restriction is achieved by limiting a given B cell to functional assembly and expression of only a single HC allele and a single LC allele as Ig chains that associate to form surface antigen receptor. This phenomenon, termed allelic exclusion (*5*), may be a unique property of the family of genes that encode antigen receptors. The total set of antigen-binding specificities (the "repertoire") expressed in newly generated B cells may be different from the repertoire expressed by subsets of peripheral B cells (*6*). Elucidation of molecular and cellular factors that influence development of these repertoires is of fundamental importance to understanding normal and aberrant immune function. This article will focus, primarily, on the aspects of the Ig variable region gene assembly process which influence the "primary" repertoire expressed by newly generated B lymphocytes, but will also consider mechanistic or selective forces that may modify the repertoire in subsets of peripheral B cells.

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Tumor Cell Model Systems

Tumors and cell lines representative of various stages of the B lineage (Fig. 1) have been used to elucidate the mechanism, control, and consequences of the Ig gene assembly process; insights provided by these systems often have been confirmed or extended by studies of corresponding normal cells (*7, 8*). We will briefly introduce

systems that will be referred to during the course of this review. Plasmacytomas and myelomas are tumors of terminally differentiated Ig-secreting plasma cells; analyses of such cells provided initial characterization of Ig structure and defined general aspects of the gene rearrangement and expression process (*9*). Fusion of normal B-lineage cells with myeloma cells generates hybridomas (*10*). Hybridomas retain the Ig gene rearrangements of the normal pre-B or B cells, thus providing a system for examining which Ig genes are rearranged and expressed in individual B-lineage cells (*11*). Transformation of fetal liver or adult bone marrow pre-B cells with Abelson murine leukemia virus (A-MuLV) generates permanent pre-B cell lines (*12*). Such lines often assemble variable region gene segments during growth in culture, demonstrating that they have an active Ig recombination system [reviewed in (*4*)]. Thus, these lines offer a dynamic system to study the mechanism and progression of Ig gene rearrangement and expression events during pre-B cell differentiation. In addition, DNA constructs (recombination substrates) that contain elements that can be assembled by the Ig recombination system have been introduced into such lines; analysis of recombination events in such constructs helped further elucidate the mechanism and control of the recombination process (*13–15*).

Molecular Basis of Antibody Diversity

The variable regions of Ig H and L chains each consist of a framework of relatively conserved amino acid sequence interrupted by three regions of highly variable amino acid sequence (Fig. 2); these "hypervariable" regions interact to form the antigen-contact area of the antibody and thus are referred to as complementarity-determining regions (CDRs) (*16*). The HC variable region is encoded by the variable (V_H), diversity (D), and joining (J) segments; LC variable regions are encoded by just V_L and J_L segments (*9*). The germline V_H and V_L gene segments each encode two of the CDRs; the third (CDR3) arises from the junctional region where the component gene segments are joined (Fig. 2; see below). Features of the somatic assembly process that contribute to diversification include: (i) the multiplicity of distinct germline variable region gene elements which encode different primary amino acid sequences; (ii) the combinatorial assortment of the various V, D, and J segments; and (iii) joining mechanisms that further diversify the coding potential of the junctional regions. The combinatorial association of H and L chains provides yet an additional mechanism to further amplify the primary repertoire. Another diversification mechanism, somatic hypermutation, operates on fully assembled Ig genes (*17*).

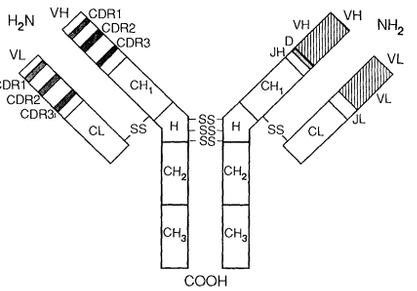

Fig. 2. The immunoglobulin molecule. A typical subunit of an Ig molecule, composed of two identical heavy chains and two identical light chains linked by disulfide bridges. Variable regions are indicated on the right subunit; CDR sequences are indicated on the left subunit.

Organization and Expression of Ig Genes

There are three families of Ig genes in mammals (HCs, κ LCs, and λ LCs); each has a distinct chromosomal location. Most studies of repertoire development have focused on HCs, primarily because the murine HC variable region locus was the first characterized in detail. Consequently, we will focus most of this review on the HC locus; general aspects of LC gene organization and expression are similar to those that we will describe for HC [reviewed in (*9*)]. In the mouse, a cluster of four J_H segments lies approximately 7 kb upstream from the first C-region gene. The nearest D segment lies approximately 1 kb upstream of the J_H cluster and approximately ten additional D segments are found within 80 kb immediately upstream; it seems that most murine D segments have been identified (*9*). The V_H gene segments lie at an unknown distance upstream of the D locus (Fig. 3) (*9*); the exact number of segments is still debated. V_H segments characterized, thus far, have been divided into nine families on the basis of relatedness at the nucleotide sequence level (*18–20*). In several murine strains, definition of families on the basis of such homology has been further validated by the observation that the families are organized into relatively discrete clusters, although limited interspersion is observed (*18, 21–23*). This clustered organization permitted ordering of families by deletion analyses (*21*) or via recombinant inbred strains (*18*) (Fig. 4). Most murine V_H families have not been physically linked with the exception of interspersed members of the two J_H-proximal groups (*23*).

Individual V_H families have been estimated to contain from a few (S107) to as many as 1000 or more (J558) members (Fig. 4) (*22, 24*). The size of the J558 family has been studied intensively. In the BALB/c mouse, Southern blotting (the number of distinct Eco RI fragments that hybridize to a J558 probe) (*18*) and "functional" analyses (see below) (*20, 25*) suggested a family size of 50 to 100 members. However, solution hybridization and other analyses led to the estimate of a much higher number, perhaps as high as 1000 to 2000 (*24*). The discrepancy between these estimates may come from several factors including the possibility that the "complexity" of this locus (the number of unique V_H segments) may be substantially less than the total number of V_H segments; due, for example, to duplications that result in multiple copies of some family members

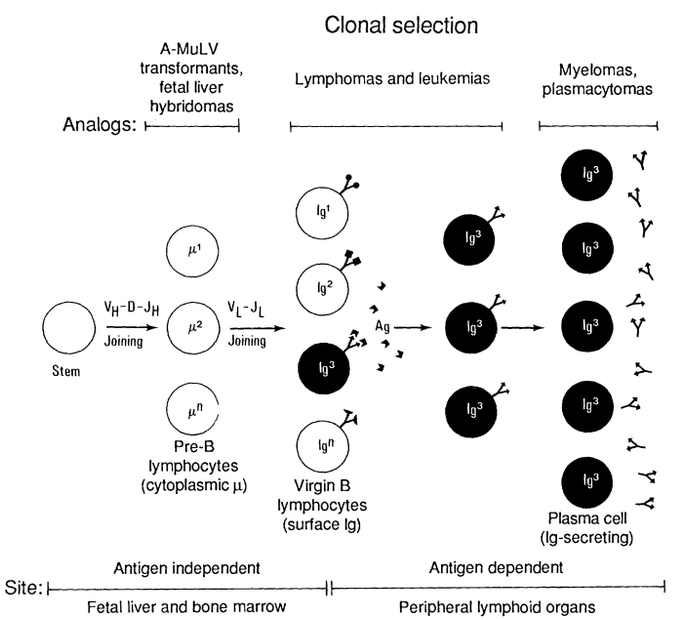

Fig. 1. Clonal selection. In primary B cell differentiation organs, stem cells give rise to surface-Ig carrying B cells during the antigen-independent phases of B cell differentiation. After migrating to peripheral lymphoid organs (such as the spleen and lymph nodes), such B cells may come into contact with cognate antigen (depicted for shaded B cell), which cause that particular cell to divide and mature into an antibody-secreting cell. Tumor cell analogs of the various stages are indicated at the top of the figure.

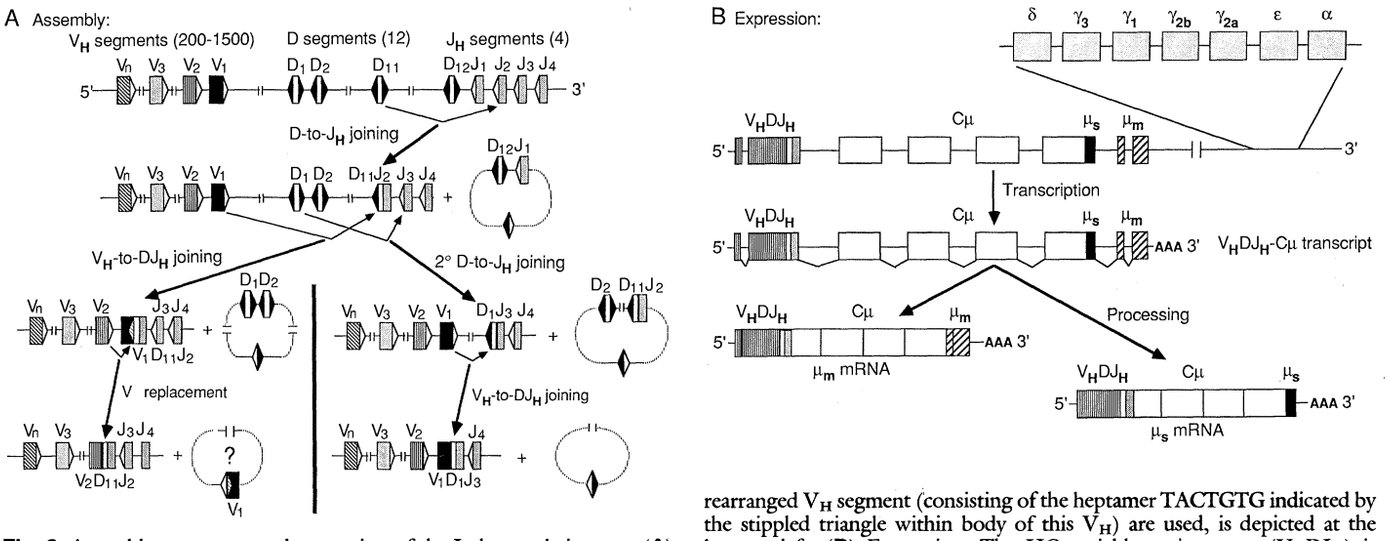

Fig. 3. Assembly, structure, and expression of the Ig heavy-chain gene. (**A**) Assembly. Assembly of V_H, D, and J_H segments. Conserved recognition sequences that lie upstream to each J_H, downstream to each V_H, and on either side of each D are indicated by triangles. Defining the 5′ end of the recognition sequence as that part which directly abuts the coding segment, this recognition sequence consists of a palindromic heptamer (related to the sequence 5′-CACTGTG-3′) followed by a spacer of 12 or 23 bp and a characteristic nonamer (related to the sequence 5′-ACAAAAACC-3′); recognition sequences with 12-bp spacers are indicated with a closed triangle in the figure, while those with 23-bp sequences are indicated with open triangles. Sequential D to J_H joining and V_H to DJ_H joining are depicted; both products of such deletional joins are indicated. Secondary D to J_H joins that can replace original DJ_H joins are shown at bottom right of figure. V-replacement, in which the normal recognition sequence of an upstream V and a partial recognition sequence encoded in the 3′ end of a previously rearranged V_H segment (consisting of the heptamer TACTGTG indicated by the stippled triangle within body of this V_H) are used, is depicted at the bottom left. (**B**) Expression. The HC variable region gene (V_HDJ_H) is assembled several kilobases upstream from the exons (open boxes) that encode the first constant region gene expressed in B cell development (C_μ). The variable region gene is divided into two exons (indicated by boxes), the smaller (upstream) exon encodes a leader sequence. Transcription initiates upstream of the V region and proceeds through the various C_μ exons; the primary transcripts can be polyadenylated downstream of a mini-exon encoding sequences specific for the secreted form of the molecule (μ_s) or further downstream beyond mini-exons encoding sequences specific for the membrane-bound form of the molecule (μ_m). The shorter primary transcript is processed to give μ_s mRNA (bottom right) where the μ_s sequences are removed from the larger primary transcript by the RNA processing mechanisms to yield the μ_m mRNA (bottom left). A series of additional constant region genes lie downstream of the C_μ gene (top right); these also have a multi-exon structure but for simplicity are indicated as single boxes.

(*24*). In addition, the absolute size of the large J558 family can vary substantially among different mouse strains (*26*), suggesting that high copy number for the family is not necessarily physiologically advantageous.

Further insight into the potential physiological significance, if any, of the organization of the murine HC locus may come from comparison with the recently elucidated structure of the human HC locus. The J_H and C_H portions of this locus have an organization similar to that of the mouse (*27, 28*) and a variety of D segments have been isolated (*27, 29*). At least six distinct families of human V_H gene segments have been identified; most are related to murine V_H families, but others are more divergent (*29–32*). Members of several V_H families, most D segments, the J_H segments, and the C_H locus have been linked by pulsed field gradient gel electrophoresis; the maximum distance between V_H and J_H segments appears to be less than 100 kb (*29*). The human V_H locus is estimated to contain several hundred members with the largest families containing fewer than 50 (*29–32*). The equivalent of the very large murine J558 family is not the largest family in humans. Perhaps the most striking contrast to murine organization patterns is that human V_H family members are highly interspersed (*29, 30*); the three largest families are interspersed over nearly the entire length of the locus (greater than 1500 kb) (*29*). This finding argues against a functional role for the distinct clustering seen in the mouse, but does not eliminate the possibility that organization of V loci evolved, at least in part, on a functional basis (*17*).

Mechanism of Variable Region Gene Assembly

Recognition sequences that mediate site-specific recombination flank all germline variable region gene segments and consist of a highly conserved palindromic heptamer, a nonconserved spacer of 12 or 23 bp, and a conserved nonamer (*9, 33*). Joining usually occurs only between segments flanked by recognition sequences with unequal spacers (referred to as the 12/23 joining rule) (*9, 33*); thus, the recognition sequences dictate which elements can join (Figs. 3A, 5). Similar or identical conserved recognition sequences flank all known Ig and T cell antigen receptor (TCR) variable gene segments and are therefore probably the target of a common site-specific recombination system, the "Ig recombinase" (*9, 14, 33, 34*). Recently, V_H gene segments have been found to recombine into the body of a downstream V_H utilized in a V_HDJ_H rearrangement. This process is probably mediated by the Ig recombinase system, which utilizes the downstream recognition sequence of the incoming V_H segment and a cryptic recognition sequence that includes a heptamer embedded in the 3′ end of most germline V_H segments (Fig. 3A) (*35*). There is no obvious nonamer sequence associated with this heptamer. The utilization of isolated heptamers for one partner of recombinase-mediated joining has been documented in other rearrangement events (*36*). Thus far, it appears that "V-replacement" is a low frequency event; whatever the frequency this process would offer yet another mechanism for junctional diversification because such events could be selected and amplified at the cellular level.

Evidence that intervening DNA frequently is lost after joining of Ig V gene elements suggested that deletion is a common aspect of the joining mechanism [reviewed in (*9*)]. However, findings that some κ-producing cells retained intervening DNA which included fused V_κ and J_κ recognition sequences ("reciprocal products") required additional explanation—leading to models of reintegration of deleted DNA (*37*), recombination between sister chromatids (*38*), and joining by inversion (*39*). Analysis of an unusual join between D and J_H segments first demonstrated that inverted joining does indeed occur (*40*); this conclusion was possible because of

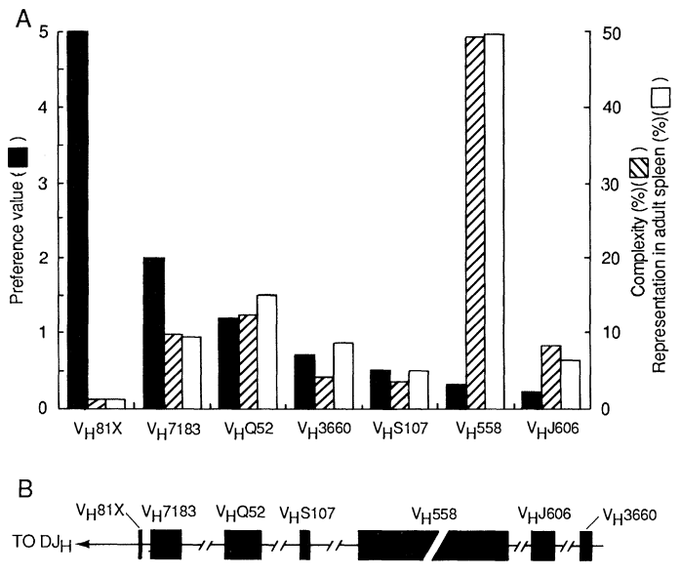

Fig. 4. The relationship between V_H gene organization and the development of the Ig heavy chain repertoire [adapted from (*80*)]. (**A**) The relative "preference" for the use of each V_H family (and the V_H81X segment) in the newborn liver compared to the adult spleen was calculated as follows: Preference = relative proportion of C_μ mRNA in newborn liver/relative proportion of C_μ mRNA in adult spleen. A value of 1 reflects equivalent representation in the newborn liver and adult spleen, whereas a value >1 indicates preferential representation in the newborn liver and a value <1 indicates relatively decreased representation in the newborn liver. Multiple independent experiments yielded very consistent results, and also revealed similar preference values for all the V_H probes used in both mouse strains analyzed; a compilation of the preference values obtained in multiple independent experiments in both mouse strains is presented. These values are compared to family complexity (*20*) and to the representation of each V_H family in the adult spleen of these mouse strains as determined by various studies (*20, 25, 80, 83*). (**B**) The organization of the murine V_H locus [adapted from (*18*)]. The relative position of the V_H families with respect to the DJ_H complex is indicated. The lengths of the boxes representing each V_H family reflect the relative number of segments in each family; the break in box representing the V_H558 family reflects the controversy concerning its size. The preferential use of the V_H families (A) is directly related to the J_H-proximity of each of the V_H families, except for the V_H3660 family; recent data suggest that this family may actually map to a more J_H-proximal location (*4*) that would be consistent with its preference number. Recent studies have suggested potential alterations of this map including placement of the 3660 family just upstream from S107 and placement of the J606 family just 3′ of J558 (*21*).

other analyses that physically linked germline D and J_H segments involved in the inversion (*41*). More recently, inverted joining of TCR V_β and DJ_β segments was documented (*42*); frequent inverted V_κ to J_κ joining has been strongly implied (*39, 43, 44*), implicating inverted joins as contributory to the retention of intervening DNA at the κ locus. Thus far, no evidence of sister chromatid exchange or reinsertion of deleted DNA has been forthcoming.

Inverted joins permit examination of both the "coding" and "reciprocal" joints (Fig. 5). The structure of the inverted HC chromosomal DJ_H joining event coupled with that of the "reciprocal" κ products, led to the proposal that joining of variable region gene segments involves a multi-step, nonreciprocal recombination process (*40*). According to this model, the process is initiated by a precise break between the elements to be joined and their flanking recognition heptamers. Subsequently, the two recognition heptamers are precisely ligated (back to back) leading either to the deletion of the intervening DNA as a circle or to its inversion, depending on the chromosomal orientation of the involved segments (Fig. 5). Joining of the coding elements was proposed to occur in a separate step, because it had long been known that coding joins were often imprecise. This imprecision is manifested by the frequent loss of original bases from one coding partner or both (V or J or both) and, in some cases, the de novo (without a template) addition of novel bases ("N" regions) between the two coding segments. The G/C–rich composition of N regions and the likelihood that they are added de novo to available 3′ hydroxyl ends after breakage between the heptamer and coding sequences was noted to be consistent with the activity of terminal deoxynucleotidyl transferase (TdT) (*40*), an enzyme found in immature B and T lymphocytes. As outlined above, modification of potential coding capacity of VDJ junctional regions by these diversification mechanisms is a significant source of antibody diversity [reviewed in (*9*)].

Studies of recombination substrates and other types of analyses have provided support for some novel aspects of this model. Inversion recombination substrates (Fig. 5) facilitated recovery of large numbers of inverted joins between V_κ and J_κ elements that were introduced into recombination-positive A-MuLV transformants (*13, 15*). Analysis of "coding" and "reciprocal" joints in the same construct clearly demonstrated imprecise ligation of coding joints accompanied by precise ligation of heptamers. In addition, recent studies clearly identified predicted circular excision products generated by heptamer fusion during deletional joining (*45*). Assuming that heptamer fusion resulting in circles is the prevailing mechanism of deletional joining, it seems possible that this recombination mechanism evolved to mediate both deletional and inversional joins; in the latter case nonfusion of heptamers would result in disastrous chromosomal fragmentations. HC variable region gene assembly appears to almost always result in deletion of intervening DNA segments, implying that HC variable region gene segments are mostly oriented for deletional joining. However, recent analyses have physically linked the human V_κ and J_κ segments and demonstrated that some V_κ segments are inverted and some are in direct orientation relative to the J_κ locus (*44*); these findings imply that inverted joining is an important pathway for κ gene assembly.

Analysis of (D to J_H) or (V_H to DJ_H) joins between endogenous segments in A-MuLV transformants supported a correlation between N-region addition and TdT activity (*46*). Recombination substrate studies, initially with thymidine kinase–based deletion recombination substrates (Fig. 5), allowed this issue to be addressed more directly (*14*). For unknown reasons, TdT expression is unstable in A-MuLV transformants. Thus, deletion substrates were introduced into TdT^+ recombination-positive pre-B cell lines; multiple TdT^+ and TdT^- subclones were derived from each of these subclonal populations and secondary subclones selected which had joined V gene segments within the construct. Analyses of the joints confirmed that bases were added between joined elements in the majority of substrate joints recovered from TdT^+ lines but in none from TdT^- lines (Fig. 5A). Because the complete structure of the substrates was known, it was confirmed that these bases were added de novo. More recently, recombination substrate experiments demonstrated preferential occurrence of N regions in substrate joints recovered from (initially TdT^-) A-MuLV transformants made to produce TdT from introduced retroviral expression vectors (*47*). Together, the different recombination substrate experiments strongly link TdT activity to N region addition. Base addition not mediated by TdT is found in joints formed by other recombination systems, raising the possibility that such mechanisms may also contribute N regions; however, N regions often seem distinct with respect to length and base-composition (*48*).

Expression of Ig Genes

All germline V_H genes carry upstream transcriptional promoter elements. However, only the promoter of the assembled V_H gene

and in some cases the proximal upstream germline segment are active in mature B cells (*49, 50*); this activity depends on a tissue-specific enhancer element found in the intron between the J_H cluster and the most proximal C_H gene (C_μ) (*51*). Germline and incompletely rearranged Ig (and TCR) constant and variable region gene segments are expressed in a tissue- and stage-specific pattern that correlates with the rearrangement of the corresponding gene segments [reviewed in (*4*)]. Transcription of germline V_H and V_L genes has been observed (*49, 52*). Likewise, D segments have transcriptional promoters; thus, DJ_H rearrangements generate DJ_H-C_μ (D_μ) transcripts which can encode corresponding HC proteins (*53*). Transcripts of most Ig HC and LC constant regions also have been noted (*54, 55*). Although most of the constant region transcripts are "sterile" (they do not encode obvious protein products), demonstrated or putative products of some germline transcripts (for example, V_H or D_μ proteins) could have an as yet to be determined function. One possibility is a regulatory role in the establishment of the repertoire (*6, 53*). Clearly, the role of germline or incomplete transcripts in normal physiology requires further investigation; however, expression patterns of these transcripts (both coding and noncoding forms) are most consistent with a relationship to regulation of gene rearrangement.

For both HC and LC, RNA processing mechanisms allow expression of the variable region on the same polypeptide chain as the corresponding constant region (Fig. 3). Sequences at the carboxyl terminus of the HC determine whether a complete Ig molecule will be membrane-bound or secreted; post-transcriptional processing mechanisms are responsible for differential expression of these sequences within the body of the C_H gene (*56*). The $C\mu$ constant region, which is the first C_H gene expressed during development, can be replaced by downstream constant regions (Fig. 3) during the maturation of a B cell; this phenomenon, termed HC class-switching (*57*), appears to be mediated by a "recombinase" system distinct from that which mediates V gene assembly. The class switch process allows clonally derived B-lineage cells to maintain the same variable region specificity in association with different HC constant regions specifying different effector functions (*17*).

Control of Ig Gene Rearrangement

The molecular and cellular basis of ordered rearrangement and allelic exclusion remain a controversial topic (*4, 58, 59*); however, certain insights have emerged. Recent evidence strongly indicates that the rearrangement process is relatively efficient and also must be regulated at some level [for review see (*4*)]. Therefore, models of allelic exclusion that suggested a high rate of aberrant rearrangement (*60*) (stochastic models) or lack of regulation of the rearrangement process per se (*59, 60*) cannot, at least in their most simple form, readily explain the allelic exclusion phenomenon. Alternative models suggest that potential protein products produced after variable region gene assembly influence further rearrangement in the cell (*61, 62*). We will focus on this regulated scheme because it best conforms to the bulk of current data; however, the possibility of other mechanisms is still open. Evidence for regulation of rearrangement comes from various sources including gene rearrangement patterns of permanent cell lines (*60, 63*), studies of A-MuLV transformants that differentiate sequentially through the pre-B stages in culture (*4*), and studies of normal pre-B and B cell populations (*7, 8*). Most recently evidence for regulation of rearrangement was obtained from studies of transgenic mice that express introduced Ig HC or LC genes (*64–66*). Recent reviews have evaluated data from cell lines (*4, 58*) and transgenic systems (*58*) with respect to control of Ig gene rearrangement; only work which extends or modifies earlier ideas will be considered in detail here.

The assembly of Ig HC variable region genes generally precedes that of LC genes during B cell differentiation (*11, 62, 67*). The first characterized step in pre-B cell development is joining of D and J_H, which usually occurs at both heavy chain alleles (*8, 46, 68*). Direct V_H to J_H joining, which would violate the 12/23 rule, has not been observed; V_H joining to D segments that have not joined to a J_H segment is very infrequent (*8*). Two types of rearrangements occur at significant frequency in cells with two DJH_H complexes (*8, 23, 68*). One is replacement of the existing DJ_H rearrangement by joining an upstream D segment to a downstream J_H segment, and the second is the attachment of a V_H segment to a DJ_H complex (Fig. 3). V_H to DJ_H joining occurs at a similar or higher frequency than DJ_H replacement (*23*). This makes physiological sense; otherwise the HC loci in most pre-B cells would "ratchet" D segments to the 3′ J_H before V_H addition, decreasing potential diversity achieved through combinatorial association mechanisms. Given that DJ_H rearrangements occur on both chromosomes before V to DJ_H joining, the above findings suggest the recombination process is somehow modified to more preferentially utilize segments from the V_H locus rather than the D locus subsequent to DJ_H formation.

The production of a μ protein in a pre-B lymphocyte is believed to mediate HC allelic exclusion by signaling the cessation of further V_H to DJ_H rearrangement in the cell and as a result, preventing the production of two different HCs (*62*). Because of imprecision in the joining process (see above), one of three V_H to DJ_H joining events should result in placement of the initiation codon of the V_H segment in the same (triplet) translational reading frame as the J_H segment and the C_μ exons (referred to as a productive rearrangement); A-MuLV transformants and normal B lineage cells appear to assemble productive HC or LC variable regions at about this predicted frequency (*7, 8, 67*). The other two-thirds of the fully assembled HC alleles contain "nonproductive" rearrangements that usually generate normal-sized HC messenger RNAs (mRNAs) which fail to encode complete HC proteins because of missense or nonsense sequences downstream of the V_HDJ_H junction (*54, 61, 63*). Given a maximal productive rearrangement frequency of 33% and two potential chances for rearrangement (two alleles), more than 60% of differentiating pre-B cells should make a productive V_HDJ_H rearrangement (*8*).

Further progression of pre-B cells beyond the V_H to DJ_H joining stage may depend on the production of HC protein. Thus, it is postulated that the μ heavy chain also can mediate onset of LC variable region gene assembly as well as HC allelic exclusion (*62, 67, 69*). In this regard, analyses of A-MuLV transformed pre-B cell lines which had two nonproductive V_HDJ_H rearrangements demonstrated that only subclones which acquired μ chain expression—either from introduced HC expression vectors or as a result of a V_H gene replacement which corrected the reading frame of a nonproductive HC allele—also actively rearranged endogenous κ LC genes and ceased to rearrange HC genes (*69*). Whether V replacement is a physiological mechanism to "salvage" normal pre-B cells with two nonproductive rearrangements is unknown, but seems unlikely if this is a low frequency event. Recent transgenic mouse studies and studies of A-MuLV transformants suggested that the membrane, but not secreted, form of the HC was effective in leading to LC rearrangement and turning off HC rearrangement (*65, 69*). Other recent transgenic mouse studies suggested that Ig δ HC (*66*) is also effective in mediating such signals. These differential activities may correlate with the structure of the COOH termini of the chains (*65, 66, 69*). B-lineage cells that produce HC in the absence of LC are known to exist, suggesting non-HC–dependent pathways for activation of LC rearrangement (*58*). However, most such lines have HC gene rearrangements and, where examination was possible, many

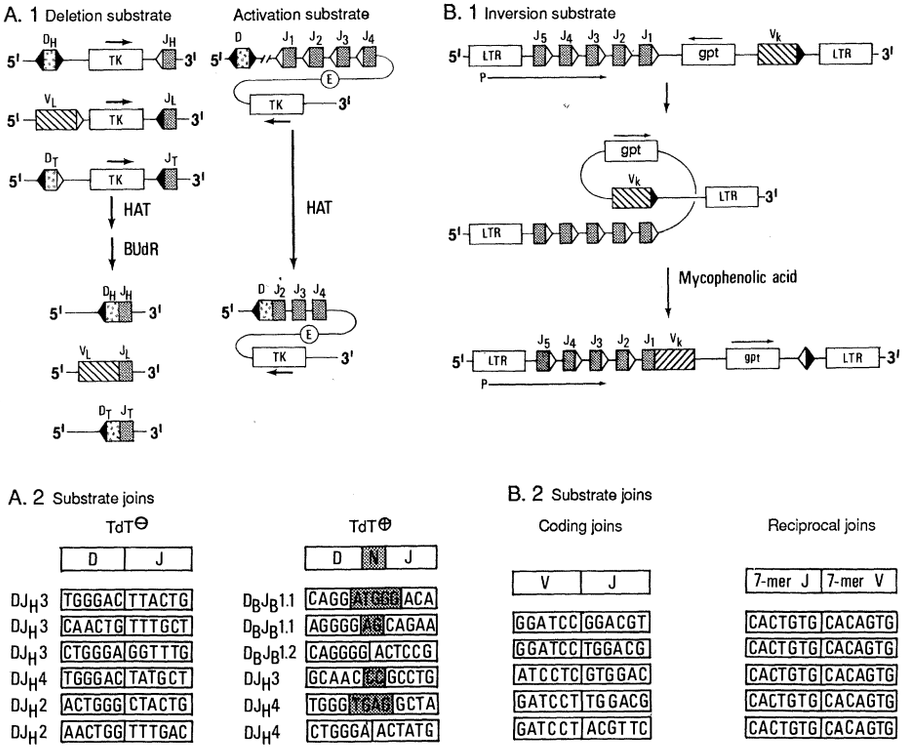

Fig. 5. Recombination substrates for analyses of the mechanism and control of variable region gene assembly. (**A.1**) Deletion and activation substrates. Deletion substrates consisting of either heavy chain D and J_H segments, λ light chain V and J segments, or TCR_β chain D and J segments flanking either side of the herpes virus TK gene are diagrammed [adapted from (*14*)]. One type of activation substrate consisting of a HC D segment, the four J_H segments, the HC enhancer and a herpes virus TK gene lying outside of this complex is also shown. These substrates were introduced into TK^- derivatives of recombination-positive A-MuLV transformants along with a second dominant marker gene. The introduced TK gene is not initially transcribed in most cells of the co-transformed population (*14*). After establishment of clonal isolates that had integrated a single or a few copies of the substrate, variants were selected in which TK was expressed. Subsequently, cells with activation substrates were directly assayed for substrate rearrangements; cells that rearranged deletion substrates were identified by selection against TK expression. (**A.2**) Analysis of substrate joins. The constructs mentioned above were introduced into recombinase-positive subclones that also expressed substantial levels of TDT. These lines were subcloned to obtain TdT^+ and TdT^- isolates. Rearranged constructs were molecularly cloned and the nucleotide sequence of the joints determined. All joints derived from TdT^- cells contained contributions only from the involved D and J segments (open boxes), whereas most joints derived from TdT^+ derivatives contained N regions (shaded boxes) between the joined D and J segments (*14*). (**B.1**) Inversion substrates [adapted from (*13*)]. The inversion substrate depicted contained the κ light chain J cluster and a V_κ gene, oriented as diagrammed, flanking either side of a promoterless bacterial guanosine phosphoribosyl transferase (gpt) gene; this cassette was inserted between two retroviral long terminal repeats (LTRs) so that the gpt gene was in the opposite transcriptional orientation from the LTR transcriptional promoter (*13*). Such inversion substrates demonstrate that recombinase-mediated joining between elements whose recognition sequences point in the same direction leads to the inversion of the DNA segment between the involved elements. Thus, joining of the V_κ to J_κ in this substrate will invert the gpt gene and allow it to be properly transcribed from the LTR promoter. The diagrammed construct was packaged into a retrovirus and used to infect recombination-positive A-MuLV transformants. (**B.2**) Substrate joins (*13*). Independent subclones expressing the gpt gene were selected and the nucleotide sequence of both the coding and reciprocal joints determined. In most of the joints the point of V_κ to J_κ joining varied (coding joins) but the involved heptamers were precisely fused (reciprocal joins).

were found to once produce HC (*8, 39*).

The separation of the HC and LC variable region gene assembly processes during B cell differentiation may be important for regulation of allelic exclusion. Production of complete Ig molecules is the putative signal that causes cessation of further rearrangement and leads to light chain isotypic (κ versus λ) and allelic exclusion (*61, 64*); sequential V_L to J_L joining at each locus apparently allows the cell to test each V_LJ_L rearrangement for production of an LC that can pair with preexisting cellular HC to make a complete Ig. In addition, this separation may allow qualitative differences with respect to HC and LC junctional diversification. Thus, N regions are rarely found in endogenous V_LJ_L joints, yet the LC V gene segments are capable of accepting N regions (*47*). TdT levels appear much lower during the stages of LC, as opposed to HC, gene assembly; thus, stage-specific assembly of HC and LC V genes coupled with stage-specific expression of TdT could lead to N-region addition to the third CDR of HC but not to LC variable regions (*40*).

The assembly of V genes from the κ LC appears to precede that from λ (*60, 61, 70*); in support of such an order, A-MuLV transformants that rearrange LC genes rearrange κ genes either exclusively or at far greater frequency than λ genes (*23, 39, 62*). In λ-producing cells both κ constant region genes are frequently deleted by recombinase-mediated joining of recognition sequences lying 3′ to C_κ to an isolated heptamer within the J_κ-C_κ intron, suggesting a role for the C_κ deletion process in the onset of λ rearrangement (*36*). On the other hand, dual κ and λ expression was found in individual B cell hybridomas made from mice transgenic for κ and populations of double-producing cells were identified in normal mice (*71*). Such results are more consistent with the absence of a relative order in κ and λ gene rearrangement. One rationalization for the existence of both pathways is that they occur in different 20^a B cell lineages (*71*); in this regard, a cell line derived from the $Ly\text{-}1^+$ B cell subpopulation co-produces κ and λ (*72*). The latter finding, coupled with other unexpected rearrangement events in a Ly-1 B cell line, led to the suggestion that Ly-1 B cells may not regulate Ig variable region assembly (*72*).

Molecular Mechanisms Which May Control Rearrangement

The idea that a single recombinase assembles all different Ig and TCR gene segments led to the proposition that stage- or tissue-specific rearrangement of different loci is controlled by modulating accessibility of particular substrate gene segments to the common recombinase (*49*). Initial support for this idea came from observations that unrearranged V gene segments are transcribed and in an "active" chromatin configuration during or prior to their rearrangement [reviewed in (*4*)]; an example is transcription of germline V_H genes in cells undergoing V_H to DJ_H rearrangement and lack of germline V_H expression in cells that express functional HC (*49*). In the context of this model, a μ HC would provide a dual regulatory signal by promoting "closing" of the germline V_H locus (making it inaccessible) and "opening" of the germline κ LC locus (making it

accessible) to the common recombinase. Support for this model came from recombination substrate experiments which involved introduction of thymidine kinase-based deletion constructs containing LC or TCR variable gene component segments into pre-B lines that rearrange only endogenous HC genes (*14*) (Fig. 5). Although these lines did not rearrange endogenous LC or TCR variable gene segments, the corresponding transfected segments were rearranged as efficiently as transfected HC variable gene segments [sometimes at a frequency of greater than 10^{-1} per cell per generation (*14*)] provided they were linked to an expressed TK gene which was assumed to substitute for normal factors in providing "accessibility." These constructs and others were not observed to rearrange (properly), even when accessible, in several nonlymphoid or mature B-lineage lines at a level of resolution that would have detected specific rearrangement events occurring many orders of magnitude less frequently (*14, 15*). The latter findings suggest recombinase activity may be limited to certain cell lineages and differentiation stages, perhaps minimizing undesirable low-frequency rearrangements such as oncogene translocations in cell types where Ig gene assembly is not required. At the current time, the exact molecular determinants of recombinase accessibility are unclear. However, utilization of "activation" recombination substrates (Fig. 5) in A-MuLV transformants yielded potential insights; rearrangement of the D to J_H segments was observed only in association with transcriptional activation of the flanking TK gene (*14*). It remains to be determined whether recombinational accessibility requires transcription or whether transcription is simply a manifestation of accessibility. A role for transcription in promoting recombination has been implicated in other systems (*73*).

Recent transgenic mouse studies suggest methods to further elucidate elements that control rearrangement specificity. A vector that consisted of a portion of the germline chicken Ig LC locus (V, J, and C segments) was rearranged in B cells of transgenic mice, indicating that the elements important for tissue-specific regulation were conserved in evolution and associated with the limited sequences in the construct (*74*). Analyses of a transgenic recombination substrate that consisted of TCR V_β, D, and J_β segments separated from an HC C_μ gene by a DNA segment in which the HC enhancer was either present or absent demonstrated that the HC enhancer was necessary and sufficient to obtain $D_\beta J_\beta$ rearrangements in the normal B and T cells of the transgenic mouse. However, complete $V_\beta DJ_\beta$ rearrangement occurred only in T cells (*75*). Thus, an enhancer may be necessary to achieve general access to the locus, but elements associated with the V_β gene, perhaps the promoter, provide the tissue-specificity of V_β to DJ_β rearrangement. Other transgenic recombination substrates were not found to give the tissue-specific rearrangement patterns of the corresponding endogenous gene segments (*74, 76*); the discrepancy may be related to factors such as copy number, proximity of segments to regulatory elements, or other factors. Defining elements within the various constructs involved with presence or absence of tissue-specific rearrangement should allow precise definition of the regulatory elements.

Position-Dependent V_H Utilization and the Primary Antibody Repertoire

The repertoire expressed by instantaneously generated populations of unstimulated B lymphocytes in primary B cell differentiation organs presumably has not been subjected to external forces that might select and bias it for particular antigen-binding specificities. Thus, the "primary" repertoire of such cells should reflect only constraints of the recombinational processes that assemble H and L chain variable region genes, together with constraints imposed by intracellular mechanisms that might select for expression of particular H and L chains (or their combinations). The constraints of the recombinational processes that affect the generation of the primary repertoire have been studied in detail only for the murine HC locus. Surprisingly, despite the apparent evolution of an immune system designed to maximize diversity, various studies revealed a highly biased utilization of V_H segments by differentiating pre-B cells. V_H segments from the J_H-proximal V_H family (V_H7183) were utilized preferentially in V_H to DJ_H rearrangements that occurred during culture of A-MuLV–transformed pre-B lines derived from the fetal liver of BALB/c mice; in fact, the J_H-proximal member of this family (V_H81X) was used at highest frequency (*77, 78*). Likewise, a pre-B line derived from the adult marrow of an NIH Swiss mouse preferentially utilized V_HQ52 segments, the J_H-proximal segments in that strain (*23*). These findings were supported by analyses of V gene usage in hybridomas from fetal liver and fetal spleen (*6, 77, 79*). Most recently, position-dependent V_H utilization was observed in the V_H gene expression patterns of different B cell differentiation organs at different times in ontogeny (*80*). The latter studies demonstrated that all V_H families are utilized in fetal development, but that their representation (and presumably rearrangement frequency) is directly related to J_H proximity (see Fig. 4). The most simple interpretation of this position-dependent frequency of rearrangement is that recombinational machinery works, at least in part, by a one-dimensional "tracking" mechanism scanning upstream from the DJ_H complex for V_H segments (*41, 77*).

A variety of studies with different mouse strains indicated that V_H segments preferentially utilized in pre-B cells are not overrepresented in mature B cells of the normal adult spleen (*80*), in normal splenic B cell colonies (*25*), or their transformed counterparts (splenic hybridomas (*6, 20*). In splenic hybridomas, representation with respect to given gene families appears to be "normalized" to roughly reflect family complexity with no obvious position-dependent bias (Fig. 4). Thus, these data suggest that a position-dependent V_H repertoire is generated in fetal lymphoid tissues and that a "normalized" repertoire exists in peripheral B cells of the adult. The exact mechanism responsible for normalization has not been clearly elucidated. However, two general theories, not mutually exclusive, have been considered: one involves programmed changes in the generation of the primary repertoire and the other invokes cellular selection forces that operate subsequent to generation of B cells in primary lymphoid organs.

Certain immune responses appear in an orderly sequence in early development (*81*). Findings of preferential utilization of the J_H-proximal V_H segments in pre-B cell lines fueled speculations (*23, 77–79, 82*) that programmed changes in gene rearrangement patterns could account for such phenomena as well as contribute to repertoire normalization in the adult. However, the expressed repertoire in various organs where B cell differentiation occurs was found to be position-dependent and to remain constant in fetal liver, neonatal liver, and in neonatal spleen in several mouse strains until at least day three after birth. Although position-dependent, this repertoire utilized segments from all V_H families (*80*). In addition, a particular V_H segment that encodes a late-appearing specificity is rearranged in the neonate (*79*). These findings suggest that ordered appearance of the repertoire cannot be explained simply by the complete absence of rearrangements utilizing more J_H-distal segments early in development followed by subsequent rearrangement later in ontogeny (programmed rearrangement). On the other hand, preferential rearrangement may result in preferential utilization of highly represented V_H gene segments in fetal as opposed to adult responses. Furthermore, the potential contribution of preferential LC V gene rearrangement to repertoire development remains to be addressed.

In the context of repertoire normalization, cellular selection would act on primary B cells and involve environmental antigens or T cells and other endogenous antigens acting on surface antibody receptors. In this regard, overall representation of different families as determined by random selective mechanisms should be related to family complexity (that is, the number of distinct binding specificities encoded by a family) (*80*). The relationship between family size and functional family complexity would be less direct than expected if individual families contained different relative numbers of nonfunctional or redundant V_H gene segments. This type of phenomenon could, at least in part, explain discrepancies between "functional" family size estimated by analyzing the frequency at which different V_H segments occur in independent splenic hybridomas or B cell colonies (*20, 25, 83*), and "absolute" family size estimated by direct genomic analyses (*24*).

In accord with a major role for selection in normalization of an initially biased repertoire, even in the adult, V_H81X segments were utilized at a much higher frequency on the nonproductive alleles of adult splenic hybridomas than would have been predicted from their frequency on the expressed allele of similar hybridomas (*78*). A recent study of V_H utilization in B cell colonies derived from the fetal liver and adult spleen concluded that two mouse strains with similar V_H locus organization had quite different V_H usage patterns unrelated to strain-specific differences in V_H family complexity. Furthermore, this analysis did not find any V_H-utilization differences between colonies derived from the fetal liver or adult spleen (*83*). A possible rationalization for the apparent differences between these findings and others is that selective events (possibly related to physiologic forces) influence the repertoires expressed during evolution of the colonies. Elucidation of the nature of such factors may be invaluable for understanding forces that mold the spontaneous repertoire.

Programmed changes in the generation of the repertoire would involve changes in recombinational or intracellular constraints that affect the composition of the spontaneously generated repertoire (*23, 77, 79, 84*). Such changes could, in theory, be modulated with respect to developmental stage or in particular sub-lineages of B cells. One conceivable mechanism that might effect such a process would be a switch from a tracking to a nontracking recombination mechanism during development, perhaps associated with programmed appearance of novel recombinase entry sites in particular regions of the V_H locus (*23*). Another mechanism would be replacement of primary V_HDJ_H rearrangements by secondary rearrangements (*35, 77*). In support of programmed rearrangements, a set of hybridomas derived from neonatal liver utilized J_H-distal V_H gene segments at a higher frequency than hybridomas derived from fetal liver (*79*). Similarly, an A-MuLV transformant derived from the neonatal spleen of a BALB/c mouse preferentially utilized V_HQ52 and not V_H7183 segments (*82*). However, as outlined above, other studies indicate that a position-dependent repertoire is continually generated in the primary lymphoid organs of the fetus, neonate, and adult. In this regard, pre-B lines derived from adult marrow that had rearranged V_H genes at the time of isolation displayed a preference for J_H-proximal V_H segments, although the preference was less striking than observed for adult marrow or fetal liver–derived lines that rearranged V_H genes during culture (*23, 77*). Furthermore, the expressed repertoire of normal adult bone marrow cells has position-dependent biases relative to that of adult spleen, although again not as striking as that of fetal liver (*80*). The latter findings are compatible with the operation of both the selection and programmed mechanisms, but also could be explained by selective forces operating on cells shortly after the rearrangement and expression process.

Recent evidence suggests that cellular selection mechanisms (programmed rearrangements notwithstanding), perhaps related to those involved in normalization of the peripheral B cell repertoire, help to shape the developing repertoire. The repertoire of early neonatal B cells contains highly autoreactive activities that are multispecific and interconnecting (*85*). This interconnectivity is manifested by the reactivities of many antibodies produced at this time toward antibody combining sites or other determinants specific to Ig variable regions; such determinants are referred to as idiotypes and the reactive antibodies as anti-idiotypes. Anti-idiotypic antibodies may play a role in establishing the dominance of particular B cell clones with respect to several well-characterized immune responses in early development and in the adult (*86*). In this context, mice that express a particular introduced transgenic HC gene appear to have altered immune repertoires; many of their endogenously encoded antibodies now share idiotypic markers specific for the transgenic HC that are rarely present in the Igs of normal mice (*87*).

Many autoreactive or interconnecting antibodies, as well as the anomalous antibodies in the transgenic mice, use V_H genes from proximal families (*85, 87, 88*). The biased representation of proximal V_H gene segments in these antibody populations could be explained in several ways. A likely possibility is that association of J_H-proximal V_H segments with autoreactive and anti-idiotypic antibodies does not reflect binding specificities unique to proximal segments, but rather reflects incidentally frequent representation of these segments in the repertoire of primary B cells or in particular B cell subsets that generate the antibodies (*80, 88*). Such cells may not have been subjected to selection mechanisms that remove autoreactivities and concomitantly result in repertoire normalization. Alternatively, evolutionary processes may have ensured that V_H gene segments encoding autoreactive or anti-idiotypic specificities required to shape the developing repertoire are preferentially rearranged early in development (*17*). A more thorough analysis of expressed V_H and V_L repertoires during development, in different B cell subsets, in different responses, and among different species may determine whether preferential rearrangement (and utilization) of V genes merely represents a necessary byproduct of the rearrangement process that must be overcome to express a normalized, maximally diverse, peripheral repertoire or whether expression of preferentially rearranged V_H segments serves an important function. In the latter context, it is notable that V_H segments preferentially rearranged in humans are related to V_H segments preferentially rearranged in most mouse strains (*32*).

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