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The Role of Somatic Hypermutation in the Generation of Antibody Diversity

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The immune system is capable of establishing an enormous repertoire of antibodies before its first contact with antigen. Most antibodies that express germ-line sequences are of relatively low affinity. Once antigen enters the system, it stimulates a somatic mutational mechanism that generates antibodies of higher affinity and selects for the expression of those antibodies to produce a more effective immune response. The details of the mechanism and regulation of somatic hypermutation remain to be elucidated.

IN ORDER TO DEAL WITH THE MANY TOXIC SUBSTANCES AND pathogenic organisms in the environment, higher organisms have developed an immune system that can react with specificity and flexibility by producing antibodies to a seemingly infinite array of antigenic stimuli. Through a process of somatic assembly of germ-line genes, an individual can produce a sufficiently large and diverse repertoire of antibody molecules to react with all of the antigens in the environment. This repertoire, however, is not sufficient to protect an organism fully, because many of the germ line–encoded antibodies have relatively low affinities. Since antibodies play their major role in the circulation by neutralizing antigens such as toxins or virus molecules that have high-affinity cellular receptors, the ability of an organism to protect itself from environmental antigens often requires antibodies that are of higher affinity. Such antibodies are generated by hypermutation of the germ line–encoded antibody genes. In this article, we focus on current knowledge of the hypermutation process and describe those aspects of the process that remain to be elucidated.

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Establishing the Antibody Repertoire

The antibody molecule consists of two identical heavy (H) chains and light (L) chains that are disulfide-linked to form a bivalent H_2L_2 molecule (Fig. 1A). The amino terminal end of each chain is encoded by a variable (V)-region gene and the carboxyl terminal end is encoded by a constant (C)-region gene. The antigen binding site of the molecule is formed by the V-region domains and differs significantly among antibodies that react with different antigens. The V-region domain contains three areas of exceptional amino acid sequence variability called complementarity-determining regions, which are the sites of antigen contact. Surrounding the complementarity-determining regions are areas of less sequence variability called framework regions. The V-region domains of the protein are encoded by a series of smaller genetic elements designated variable (V_H), diversity (D), and joining (J) for the heavy chains and V_L and J_L for the light chains (Fig. 1A). Each genetic element is part of a larger linkage group, and those encoding the murine heavy and both light chain [kappa (κ) and lambda (λ)] V regions are located on different chromosomes. Each group of genetic elements is separate in the germ-line configuration (Fig. 1B), and for the heavy chain there are 100 to 1000 V_H (*1, 2*), approximately 12 D, and 4 J genetic elements. In the mouse, the κ light chain has more than 100

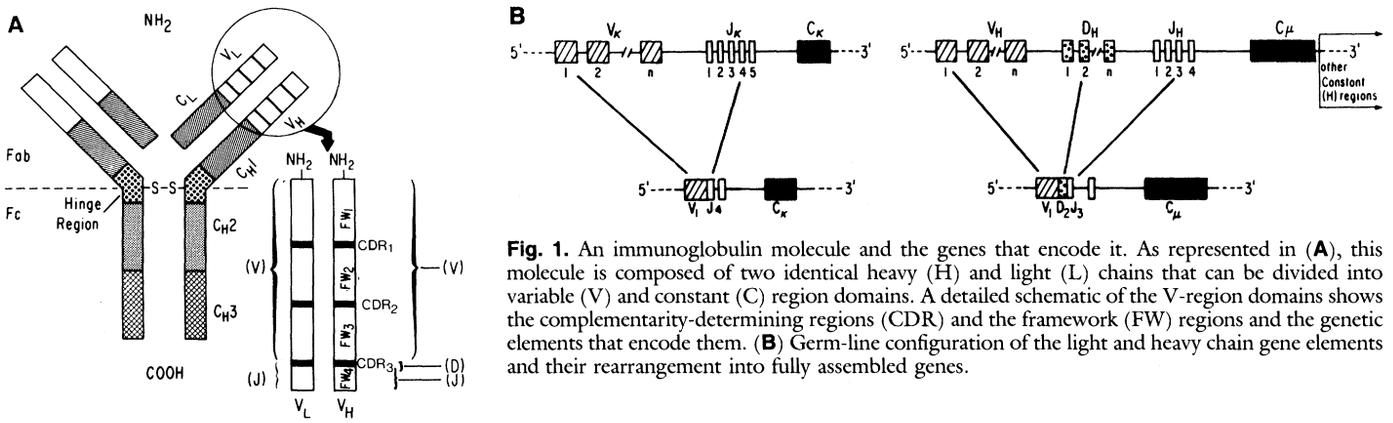


Fig. 1. An immunoglobulin molecule and the genes that encode it. As represented in (**A**), this molecule is composed of two identical heavy (H) and light (L) chains that can be divided into variable (V) and constant (C) region domains. A detailed schematic of the V-region domains shows the complementarity-determining regions (CDR) and the framework (FW) regions and the genetic elements that encode them. (**B**) Germ-line configuration of the light and heavy chain gene elements and their rearrangement into fully assembled genes.

V and 5 J genetic elements, one of which, $J_\kappa 3$, is a pseudogene.

The formation of a complete V region occurs by a site-specific recombination mechanism (Fig. 1B) (*1, 3*). Sequence diversity generated by the association of different combinations of the numerous heavy and light chain V gene elements is further increased by slightly varying the recombination site, thus generating different codons at the junction between each of these gene segments (*1*). In the heavy chain, additional sequence diversity is created at the VD and DJ junctions by the enzymatic (*4*) introduction of bases, called N sequences, that are not found in the germ line. The crucial point is that these combinatorial and junctional mechanisms of rearrangement as well as the combinatorial association of different heavy and light chains provide enormous sequence diversity and can generate 10^8 to 10^{10} different antibodies from a relatively small amount of genetic information (*1*).

Before each of the many antibodies can be presented on the surface of individual antibody-forming B cells, the V-region genetic elements are rearranged and brought into proximity with their respective C-region genes, a process that triggers their expression. Processing of pre-mRNA then joins the V- and C-region genes to produce mRNA that is translated into the complete polypeptide chains. The heavy chain genes rearrange and are expressed first in the cytoplasm of the cell along with the μ C-region gene, and this is followed by the rearrangement and expression of a light chain (Fig. 2A). Only after both chains are expressed in the cytoplasm will a complete antibody molecule appear on the cell surface. In each antibody-forming cell, only one heavy chain and one light chain V region are productively rearranged and expressed (allelic exclusion). The V region of the other allele can also be rearranged; however, because of aberrancies in the rearrangement process this allele is nonproductive. This process results in the presentation on the surface of individual B cells of an antibody with a single antigen specificity. Those cells producing the antibody of highest affinity can then be selected by antigen for further proliferation.

Extensive somatic mutation of the rearranged V-region genes results in the production of higher affinity antibodies. This process is triggered when B cells expressing low-affinity surface antibody are stimulated by antigen to interact with T cells and antigen-presenting cells. Growth and differentiation factors resulting from this interaction induce the proliferation and further differentiation of the antibody-forming cell (*5*). After interaction with antigen, the B cell can follow a number of different pathways (Fig. 2A). It can terminally differentiate into an immunoglobulin M (IgM)–secreting plasma cell; it can switch (*6*) its fully assembled V region to a downstream C-region gene to express and secrete a new class of antibody with a different C region that is associated with a specific effector function (7); or it can become a memory cell that can be recognized and restimulated by the same antigen to proceed through these same pathways (Fig. 2, A and B). Some have suggested that the memory cell may be the target cell for the hypermutation of rearranged heavy and light chain V-region genes (*8, 9*). This process is depicted in Fig. 2B by the accumulation of mutations in the V region (X and Y) as B cell development continues. Even though heavy chain class switching and hypermutation appear to occur at similar times during B cell differentiation and require T cell help (*5, 10*), these events occur independently and are the products of different molecular processes.

Characteristics of Somatic Hypermutation

Examination of the amino acid sequences of the λ light chains produced by mouse myeloma tumors led Weigert *et al.* (*11*) to suggest in 1970 that immunoglobulin genes undergo extensive somatic mutation. Eight years later this deduction was proved to be correct when the genes encoding the germ-line light chain were cloned and sequenced (*12*). Subsequently, comparison of the amino acid and nucleic acid sequences of immunoglobulin produced by malignant plasma cells, and of the germ-line V-region genes that encode them, revealed that most antibody molecules differed by large numbers of base changes from the germ-line sequences (*13–17*). For example, Kim *et al.* (*16*) showed that the heavy chain V region of one IgA-producing mouse myeloma differed from its germ-line sequence by 38 base changes. Many of these changes occurred in the coding region of the gene, but large numbers of mutations were also found in the immediate 5′ and 3′ flanking regions (*16–18*). In one malignant human B cell lymphoma, most of the somatic mutations were in the J-C intron (*19*). However, this instability does not usually extend into the C region (*9, 15, 16, 19–21*), and the focus of mutation is in and immediately around the coding sequences of heavy and light chain V regions.

Although the in vivo studies on immunoglobulins produced by malignant antibody-forming cells have been informative, even more has been learned from the studies of families of monoclonal antibodies generated in response to a single antigen, when the variable regions of the antibodies are encoded by one or a few germ-line genes (*9, 22–28*). The availability of hybridomas making such antibodies has made it possible to compare the sequences of large numbers of closely related antibodies that (i) arise in the course of the immune response in a single animal and are all deduced to be the products of a single antibody-forming B cell clone (*23, 26, 28–31*); (ii) arise in different animals but at the same time in the immune response and at the same stage during B cell differentiation (*9, 22, 26, 31–33*); and (iii) are part of the response to a single antigen but

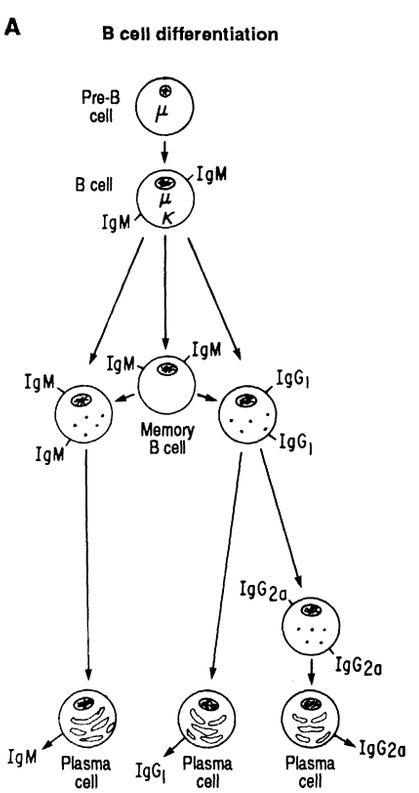
are encoded by different germ-line genes (*9, 22, 24, 32, 33*).

The analysis of the nucleic acid sequences of such monoclonal antibodies has confirmed that the rearranged heavy and light chain V-region genes accumulate large numbers of base changes during the course of the immune response. The molecular basis of these events is still completely unknown. Because there are families of closely related germ-line V-region genes (*34*), it is sometimes difficult to be certain whether an apparent mutation occurred de novo or represents the expression of a similar germ-line sequence that resulted from recombination or gene conversion (*35*). This issue arose because the chicken light chain V-region repertoire is established by gene conversion from a large library of pseudogenes that donate sequences to a single functional downstream V-region gene (*36*). Furthermore, in cultured mouse cells, an expressed V-region gene can be replaced by an upstream V-region gene (*37*). To examine the role of gene conversion or V gene replacement during the immune response in mice, we constructed oligonucleotide probes that contain single base changes that are unique for a particular monoclonal antibody and showed that these base changes do not exist either in other germ-line V regions or in isolated donor sequences larger than 25 base pairs elsewhere in the genome (*20*).

The examination of the sequences of monoclonal antibodies expressing a well-characterized germ-line V region suggests that the base changes that appear during the immune response are generated by a hypermutation mechanism (*9, 20, 22–33*). This hypothesis is supported by many studies in which sets of monoclonal antibodies that are all the product of a single B cell clone are analyzed by organizing them into a genealogical tree (*23, 26, 28–31*). Their common clonal origin is deduced from the fact that they have the same heavy chain VDJ and light chain VJ, the same heavy chain VD or DJ junctional sequences, including N sequences, and identical nonproductive immunoglobulin alleles (*23, 26, 28–31*). The genealogical tree is constructed by arranging the individual antibodies in accordance with shared and unshared base changes in the nucleic acid sequences of each monoclonal antibody (*23, 29*) (Fig. 3). The construction of such genealogical trees may be complicated by the fact that similar mutations occur repeatedly even in antibodies from different animals, an indication of "hot spots" and selection (*9, 26, 31*). Nevertheless, the appearance of additional base changes in successive branches of a genealogical tree indicates that single base changes, or small groups of changes, occur sequentially during successive cell divisions.

The analysis of the point mutations in many families of related monoclonal antibodies from different strains of mice and of the genealogies created from the monoclonal antibodies derived from individual animals led to calculations of mutation rates of 10^{-3} to 10^{-4} changes per base pair in a cell division (*9, 23, 27–32*). Although these calculations are the best estimates that can be made from the available in vivo data, they are usually based on the assumptions that the cells are doubling every 17 to 18 hours and that the process of somatic mutation is continuously active. However, the doubling time of antibody-forming cells may be less than 10 hours (*38*), thus decreasing the calculated rate, whereas the occurrence of somatic mutation during a brief time in B cell development would greatly increase the rate. Since the antibody-forming cells expressing the highest affinity antibodies are selectively stimulated to proliferate, the possibility exists that the calculated mutation rates are based largely on those cells that have undergone the highest rate of mutation. This possibility would bias the calculations toward a higher than actual mutation rate, even if the calculation is based only on the number of silent mutations in cells that were selectively stimulated to proliferate. In spite of these problems, the mutation rate in the immunoglobulin V regions is certainly several orders of magnitude higher than the estimated rates of mutation for other genes in antibody-forming cells (*39*). The calculated rates of 10^{-3} to 10^{-4} changes per base pair in one generation for the immunoglobulin genes are close to the theoretically optimum rates to provide maximum sequence diversity without completely destroying the protein structure (*26, 40*).

Attempts have been made to deduce the mechanism of somatic hypermutation from the nature of the mutations and their surrounding sequences. Since all possible changes are observed at reasonable frequencies, the chemical nature of the changes has not been informative. A number of mechanisms have been proposed. These include an error-prone repair process utilizing either a special enzymatic system, reverse transcription, or perhaps an unusual modification or combination of the usual repair systems that would selectively recognize a DNA sequence in rearranged heavy and light



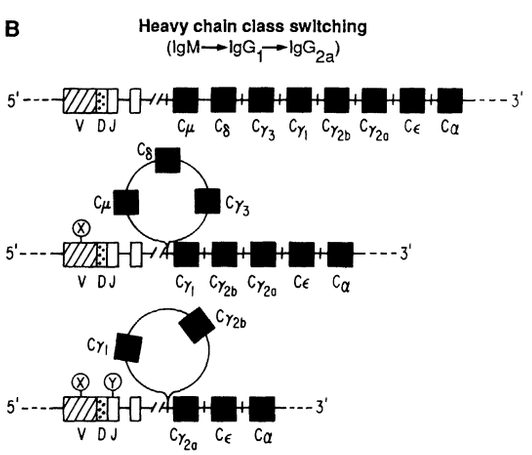


Fig. 2. The developmental stages of a B cell differentiating into an antibody-secreting plasma cell and the molecular events associated with immunoglobulin class switching and somatic hypermutation. (**A**) B cell differentiation begins with a pre–B cell producing cytoplasmic μ heavy chains. The production of light chains results in the surface expression of a complete IgM molecule. The subsequent stages of B cell differentiation are schematically shown and discussed in the text. (**B**) The heavy chain C region genes are associated with a rearranged V region. The C regions encoded by each of these genes carry out specific effector functions such as binding to Fc receptors on phagocytic cells or mast cells, passing through the placenta or into secretions, and fixing complement. The deletional mechanism of heavy chain class switching is represented opposite the relevant antibody-producing cells shown in (A). Somatic hypermutation that occurs during B cell differentiation is represented by X and Y in the V region.

chain V regions and cleave and digest it, and introduce errors by unfaithful repair (*41*). In a modification of the same theme, short, direct and indirect repeats of almost identical sequences in immunoglobulin V regions could form mismatched palindromes that could be used as a template for an error-prone replication or a repair process that would introduce mutations (*42, 43*). In one analysis, many of the V-region mutations occurred in such palindromic sequences (*43*). However, it is not clear that such palindromes are actually formed; furthermore, similar palindromes are also present around the C-region gene (*43*). While mechanisms such as these would explain hot spots (*9*) and apparent clustering of point mutations (*17, 19*), there must be additional factors to explain the restriction of the hypermutation to the V region and its immediate flanking sequences, its occurrence during a particular period in B cell differentiation, and the lack of somatic mutation in the T cell receptor that is assembled from similar genetic elements.

The high rate of somatic mutation seems to act concomitantly on the heavy and light chain V regions expressed initially in response to antigen, and these V regions often continue to dominate the later phases of the response. As a result of progressive somatic mutation, antibodies with increasingly higher affinity can be created, and those B cells producing them will be selectively stimulated to proliferate and differentiate by antigen (*9, 22, 24, 26, 29–32*) (Fig. 3). The role of affinity maturation (*44*) and antigen selection have been suggested by studies demonstrating that a single amino acid substitution in a complementarity-determining region can result in a tenfold increase in affinity (*9, 45*). The potential of somatic mutation to produce progressive increases in affinity has been confirmed by the use of site-specific mutagenesis to reconstruct antibodies that represent a genealogy that undergoes affinity maturation (*46*).

In response to some antigens, B cells expressing an initial dominant heavy and light chain V-region combination are partially replaced by other B cell clones expressing different germ-line V regions (*9, 22, 33*). When these new V-region combinations appear in the immune response they have undergone extensive somatic mutation (*9*). These V-region combinations may not have been prominent early in the immune response because their germ-line counterparts did not have sufficient affinity for the eliciting antigen and required somatic mutation to acquire a high enough affinity for selection and amplification. However, their appearance also raises the possibility that an antibody that is reactive with one antigen can, as a result of somatic mutation, either undergo a significant decrease in reactivity (*8, 32, 47, 48*) or acquire high-affinity interactions with another antigen. We have seen this occur in an antibody to phosphorylcholine produced by a mouse myeloma cell line. As a result of a single amino acid substitution that occurred in vitro, this antibody acquired reactivity with double-stranded DNA, with phosphorylated proteins, and with phospholipids such as cardiolipin (*49*). The issue of why such autoantibodies do not routinely appear in the circulation has yet to be resolved.

The study of families of monoclonal antibodies all derived from one or a few germ-line V regions has made it possible to postulate when in B cell differentiation somatic mutation occurs. Milstein and his colleagues (*9, 22*) showed that antibodies of the IgM and IgG classes made during the first 7 days after immunization did not have any somatic mutations, whereas antibodies made 14 days or more after antigenic stimulation had acquired significant numbers of base changes. Since IgM antibodies undergo somatic mutation (*20, 28, 30*), heavy chain class switching is not required for this event to occur. Once somatic mutation has begun, repeated rounds must occur over the period of at least a few cell divisions, since successive antigenic challenges separated by long periods of time result in the accumulation of mutations with progressive increases in the affinity of the antibody (*9, 22*). Based on purely circumstantial evidence, the

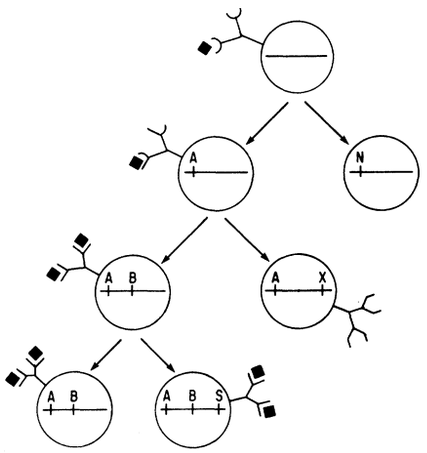


Fig. 3. The formation of a genealogical tree based on the nucleic acid sequences of monoclonal antibodies generated from a single animal. The variable region of the surface antibody expressed on the first cell is encoded by rearranged germ-line gene segments that result in an antigen-binding site of low affinity. The antigen does not fit precisely into this site. After antigen stimulation and activation of the hypermutation mechanism, mutations can be identified in the gene sequence as represented by an A (replacement mutation) or an N (nonsense mutation). The replacement mutation results in the expression of an antibody with increased affinity; thus, its binding site has a better fit for the antigen. The nonsense mutation results in a termination codon; therefore, an antibody is not expressed, and this cell does not undergo further proliferation. After further antigenic stimulation, additional mutations, B and X, appear and result in the expression of antibodies with high affinity (A and B) or a loss or change of antigen specificity (A and X). The high-affinity antibody has a binding site with a precise fit and higher affinity for antigen, illustrated by the occupation of both antibody binding sites. These antibody-producing cells are presumed to be derived from the previous cell because of the similarities in the sequences, including point mutations, junctional sequences, and nonproductive alleles. Mutations continue to accumulate during the immune response and silent mutations that do not result in amino acid substitution, represented by S, can occur at any time.

suggestion has been made that this occurs in the memory B cell compartment (*8, 9*) in the germinal centers of lymphoid organs (*9*). As already noted, the appearance of additional unique somatic mutations in different antibodies originally derived from the same clone of B cells means that all of the base changes do not occur during one cell division. It is likely that somatic mutation is turned off as the B cell differentiates into a plasma cell that is secreting large amounts of antibody. The rearrangement of the VDJ from IgM to IgG C regions could result in the loss of DNA sequences that are signals for somatic mutation (*50*), a proposal that is supported by studies in which B cells that have already undergone significant somatic mutation do not continue to accumulate somatic mutations, even though they continue to proliferate and switch to downstream C regions (*8, 30*). These in vivo studies, as well as studies of cultured cells, have led to the belief that somatic hypermutation of immunoglobulin V regions occurs during a particular period in B cell differentiation. Since hypermutation occurs in the heavy and light chain V regions, in the productively and nonproductively rearranged V regions (*13*), as well as in transfected genes of transgenic mice (*51*), it seems likely that a trans-acting mutagenic system is activated for a brief time.

Analysis of Somatic Hypermutation in Vitro

Although a great deal has been learned from the sequences of antibodies that reflect mutational events in vivo, these antibodies have passed through many levels of selection, and one cannot be certain that the impressions we have are all correct. If we are to learn more about the regulation and detailed molecular mechanism of hypermutation, systems must be developed for further dissection of these processes. Ideally, we would like to generate antibody-forming cell lines that will undergo V-region hypermutation in culture. An in

vitro system would allow true mutation rates to be determined, unselected products of the mutational mechanism to be examined, the role of T cell or other factors to be studied, manipulated genes to be transfected into cells to identify the DNA sequences that act as signals for mutation, and ultimately the cloning of the genes that are involved.

The question of hypermutation in antibody-forming cells was initially examined in vitro by adapting mouse myeloma cells to tissue culture and identifying mutant antibodies. This analysis was successful in that spontaneous immunoglobulin mutations were identified at frequencies of approximately 0.1%. However, the mutations occurred in the C region rather than the V regions (*52*). Adetugbo *et al.* (*53*) used isoelectric focusing to analyze the immunoglobulins secreted by 7000 independent subclones of a mouse myeloma cell and found four variants that had point mutations or deletion and frameshifts in their heavy chain C regions. We made similar observations by cloning cells in soft agar and overlaying with antibodies against the secreted immunoglobulin (*54*). These results prompted the use of more sophisticated techniques to attempt to identify specifically V-region mutants. Kohler and Shulman (*55*) created a selection assay in which they bound hapten to the surface of hybridoma cells that were producing IgM antibodies specific for that hapten. In the presence of complement, the secreted IgM bound back to the surface of the hybridoma cells and lysed them so that only those cells producing mutant antibodies survived. All of the mutant antibodies that were studied in detail had structural changes in their C region that led to a loss of complement fixation or blocks in assembly (*55, 56*). Although a few mutants produced intact immunoglobulin with a decrease in antigen binding (*55, 57*), in the absence of sequence data, it is not possible to know whether those mutants had changes in their variable regions.

More recently, we developed a detection system for identifying rare variants with increased binding of antigen (*58*). Spontaneous higher binding mutants arose at a frequency of 10^{-5}, but of the seven independent mutants sequenced, none had a single base change in the heavy or light chain V regions and all had changes in their C regions, including point mutations, deletions, and frameshifts. All of these changes led to increases in avidity but not to changes in affinity. Matsuuchi *et al.* (*59*) identified polysaccharide-binding hybridomas that generate antigen-binding mutants at a relatively high frequency, but they have shown that these changes are due to mutations in glycosyl transferases that affect the structure of carbohydrates in the antigen binding site and not to amino acid substitutions. These and other studies suggest that in myelomas and hybridomas the frequency of mutations in the V region is extremely low. This has been confirmed by Rajewsky and colleagues (*60*), who identified hybridoma cells producing immunoglobulins with changes in their variable regions at a frequency between 10^{-6} and 10^{-8} and showed that these mutants had point mutations and, in one case, a possible gene conversion. Panka *et al.* (*47*) identified rare spontaneous variants with changes in their relative ability to bind two closely related antigens and showed that these variants were the result of mutations in their V regions.

The available evidence suggests that hybridoma and myeloma cells in culture undergo V-region mutations at low frequencies comparable to those seen in other genes and that C-region mutations and deletions occur at frequencies in the range of 10^{-5}, which are high but not as high as the apparent rate of V-region mutation in vivo. This paradox raises the possibility that highly differentiated antibody-forming cells have actively turned off V-region hypermutation so that they will continue to produce the high-affinity antibodies whose expression was selected for earlier in B cell differentiation.

In fact, only two cultured cell lines were shown to undergo somatic hypermutation of their endogenous V-region genes. The S107 mouse myeloma cell line generated heavy chain V-region mutations at spontaneous frequencies of approximately 0.1% (*61*). In the two mutant genes that were sequenced, point mutations in the heavy chain V region resulted in a loss of antigen binding (*48*). The S107 immunoglobulin gene had one or two base deletions in the C region at similar frequencies, an indication that the instability in this myeloma cell line is not restricted to the V region (*62, 63*). Moreover, in recent experiments we were unable to identify additional V-region mutants, perhaps because the expressed gene is duplicated and the expression of both genes may mask new mutations that arise (*63*).

One clone of the 18-81 Abelson transformed pre–B cell line has been observed to undergo somatic V-region mutation frequently (*64*) and appears to be a better model for the normal process. This cell line has two rearranged VDJ heavy chain alleles but has not progressed to the stage of light chain rearrangement. One of the V regions is rearranged to the μ constant region but contains a nonsense mutation that results in a lack of translation. Wabl and colleagues (*64*) have used the expression of the μ C region to determine the rate of mutation, by reversion analysis, of the nonsense mutation. This rate was calculated to be 10^{-5} changes per base pair in one generation (*64*), which is high but lower than the estimated rate of V-region hypermutation in vivo. The difference may be due to a lack of T cell factors or may occur because 18-81 is less mature than the memory B cells that are normally thought to undergo somatic mutation. However, it is also possible that the in vivo rate has been overestimated and that 10^{-5} changes per base pair in one generation really reflects the normal rate. Of importance is the fact that C-region mutations occur in 18-81 at a much lower rate (*65*). Furthermore, when 18-81 is fused to one of the myeloma cell lines commonly used to make hybridomas, V-region mutation is reduced (*64*). Although only one hybrid was analyzed in detail, the results support the idea that mature antibody-forming cells have an active trans-acting mechanism for turning off V-region hypermutation and could explain why hybridomas do not undergo frequent V-region mutation.

This finding also makes it clear that vectors that are potential substrates for somatic hypermutation must be transfected into cells that are permissive for somatic hypermutation. Studies with transgenic mice have shown that exogenous immunoglobulin genes can undergo somatic mutation, even though they are not integrated into the endogenous immunoglobulin locus (*51*). Since homologous recombination has been carried out with immunoglobulin genes (*66*), it may be possible to examine the role of distant and local sequences in regulating somatic mutation in transgenic mice. However, studies of the genetic and molecular mechanisms and enzyme systems and their regulation would be greatly facilitated if additional cell lines that carry out somatic mutation could be generated.

Conclusion

The generation of high-affinity antibodies depends on the somatic hypermutation of rearranged immunoglobulin V-region genes. These mutational events are restricted to the V regions and their immediate flanking sequences and probably occur during a brief time in B cell differentiation. Affinity maturation of the immune response is presumed to occur when point mutations that lead to higher affinity antibodies enable the antibody-producing B cells to interact preferentially with antigen and helper T cells and to be amplified selectively. The molecular mechanism responsible for V-region hypermutation and its regulation during B cell development is not known and the elucidation of these processes will require the development of new in vivo and in vitro systems.

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67. The work cited from the laboratory of M.D.S. is supported in part by NIH grants CA39838, AI10702, and 5P30CA13330 and in part by the Harry Eagle Chair in Cancer Research from the National Women's Division. D.L.F. received a fellowship from the New York Heart Association and has been supported by a training grant from NIAID (5T32AI07183). She is currently supported by an NIH training grant (5T32CA09173).