First published online as a Review in Advance on June 21, 2005

Immunoglobulin Gene Diversification

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Annu. Rev. Genet.
2005. 39:23–46

doi: 10.1146/
annurev.genet.39.073003.110544

0066-4197/05/1215-
0023$20.00

Key Words

antibody, deamination, mutation, recombination, repair

Abstract

Three processes alter genomic sequence and structure at the immunoglobulin genes of B lymphocytes: gene conversion, somatic hypermutation, and class switch recombination. Though the molecular signatures of these processes differ, they occur by a shared pathway which is induced by targeted DNA deamination by a B cell–specific factor, activation induced cytidine deaminase (AID). Ubiquitous factors critical for DNA repair carry out all downstream steps, creating mutations and deletions in genomic DNA. This review focuses on the genetic and biochemical mechanisms of diversification of immunoglobulin genes.

Contents

DIVERSIFICATION CREATES A ROBUST AND DYNAMIC IMMUNE RESPONSE

The immune system must recognize an immense variety of different pathogens and respond dynamically as microorganisms evolve during the course of infection. To accomplish this, lymphocytes carry out targeted and regulated alterations of genomic structure and sequence. Early in B cell and T cell development, antigen receptor variable (V) regions are constructed by V(D)J recombination, a site-specific recombination process initiated by the lymphocyte-specific proteins RAG1 and RAG2 (8, 37), as shown in **Figure 1**. Following successful V(D)J recombination, a B cell expresses an immunoglobulin (Ig) molecule composed of two distinct chains, a heavy chain and light chain, encoded at separate alleles. The variable regions of the heavy and light chains together form a domain that recognizes antigen, and the heavy chain constant (C) region determines how antigen is removed from the body. Subsequently, B cells increase the diversity of the repertoire and tailor Ig molecules to optimize the response to a specific pathogen, by modifying genomic sequence and structure of the rearranged and expressed Ig genes in characteristic and striking ways.

These processes occur only in B cells, and they are regulated by developmental and environmental stimuli. They are essential, and immunodeficiency results if they are impaired. Although the molecular signatures are distinct, all these processes are initiated by a single B cell–specific factor, activation-induced deaminase (AID). AID is a cytidine deaminase that carries out targeted deamination of C → U at expressed Ig genes. Uracil in DNA is normally repaired by conserved, ubiquitous, and high-fidelity pathways, but at the Ig genes of B lymphocytes, repair is somehow diverted to ensure a mutagenic outcome. AID is the only B cell–specific factor involved, and downstream steps depend on ubiquitous repair factors that, paradoxically, promote mutagenesis of B cell Ig genes. This review discusses our current mechanistic understanding of the pathways of Ig gene diversification.

MOLECULAR SIGNATURES OF Ig GENE DIVERSIFICATION

The signatures of Ig gene diversification are different at different loci, in different species, and in response to distinct developmental and environmental regulation. As background, three of the best-characterized examples are described below.

Gene conversion (templated mutation): produces sequence changes with matches in germline donor genes or pseudogenes

Somatic hypermutation (nontemplated mutation): produces point mutations with no matches in germline DNA

Class switch recombination: joins a new constant region to the expressed heavy chain variable region, promoting deletion of many kb of chromosomal DNA

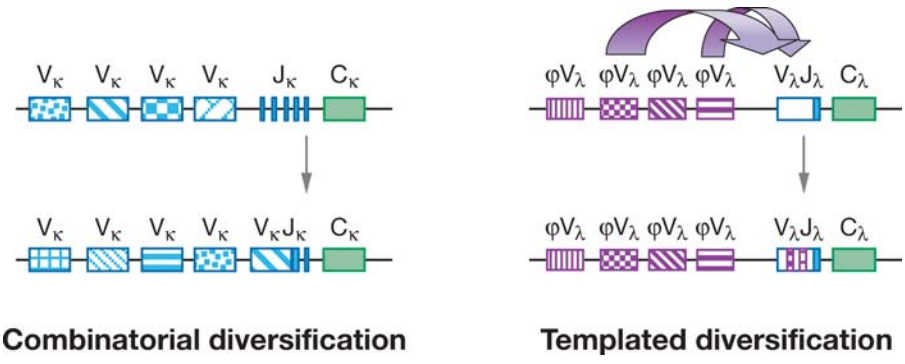

Figure 1

Combinatorial and mutagenic diversification of V regions. *Left*, combinatorial diversification at the murine κ light chain locus. In each B cell, one of many Vκ segment recombines with one of several J_κ segments to produce a functional κ variable region. *Right*, templated diversification at the chicken λ light chain locus. The $V_\lambda J_\lambda$ variable region created by recombination of the single functional V_λ and J_λ segments undergoes gene conversion templated by upstream V_λ pseudogenes (φV_λ).

Gene Conversion (Templated Mutation) of Chicken Ig V Regions

Chickens have only a single functional light chain V segment, V_λ, which recombines with a single J_λ segment very early in B cell development. The rearranged and expressed $V_\lambda J_\lambda$ then undergoes gene conversion, or templated mutation. An array of 25 nonfunctional, "pseudo-V_λ" regions, located just upstream of the functional V_λ, provide donors for sequence transfer. Diversified Vλ regions contain tracts of sequence changes with clear matches in germline DNA (110, 127). **Figure 1** compares combinatorial diversification of murine V regions and templated diversification of chicken V regions.

Somatic Hypermutation (Nontemplated Mutation) in Murine and Human B Cells

In humans and mice, a multitude of V, D, and J segments recombine to create the functional Ig genes of the pre-immune repertoire (8, 37). Following antigen activation, the expressed heavy and light chain V regions are altered by somatic hypermutation, which obliterates existing sequence information and inserts nontemplated (point) mutations (61, 64, 83). Hypermutation is targeted to the transcribed V regions; the C regions and the rest of the genome remain untouched. Hypermutation occurs following activation with antigen, and is coupled with selection for B cell clones that express high-affinity antibodies. Together, hypermutation and clonal selection not only increase antibody affinity, but also provide a dynamic response to pathogens that are simultaneously undergoing continuous mutation and selection.

Mammals produce four isotypes (or classes) of Ig: IgM, IgG, IgE, and IgA, encoded by the μ, γ, ε, and α constant regions, respectively. Related IgG subclasses are encoded by distinct Cγ regions. Each Ig isotype is specialized for particular modes of antigen removal. IgM, the first isotype synthesized by a B cell, activates complement. IgG, the most abundant isotype in serum, binds receptors on phagocytic cells. IgG antibodies cross the placenta to provide maternal protection to the fetus. IgA antibodies are abundant in secretions, such as tears and saliva; they coat invading pathogens to prevent proliferation. IgE antibodies can provide protection against parasitic nematodes, but in developed countries they are the bad guys: They bind basophils and mast cells, activating histamine release and allergy.

Class Switch Recombination in Mammalian B Cells

Class switch recombination detaches an expressed heavy chain variable (VDJ) region from one constant (C) region and joins it to a downstream C region, deleting the DNA between (19, 61, 66) (**Figure 2**). The C region determines how antigen is removed from the body, and each class of C region is specialized for clearance of specific kinds of pathogens, via specific pathways (SIDEBAR). Thus, switch recombination modifies how an Ig molecule removes antigen without affecting specificity of antigen-recognition by literally *switching* C regions. Recombination junctions form within switch (S) regions, repetitive and degenerate guanine-rich regions 2–10 kb in length. Recombination is region-specific, not sequence-specific, and produces junctions at heterogeneous sites and sequences within the S regions (29). Switch junctions are within introns, so junctional heterogeneity does not affect the sequence or structure of the heavy chain polypeptide. A dedicated enhancer and promoter upstream of each S region activate S region transcription in response to extracellular signals delivered by T cells and cytokines, which thereby regulate production of specific classes of Ig. The critical signal for recombination is transcription in *cis* of the S regions targeted for recombination.

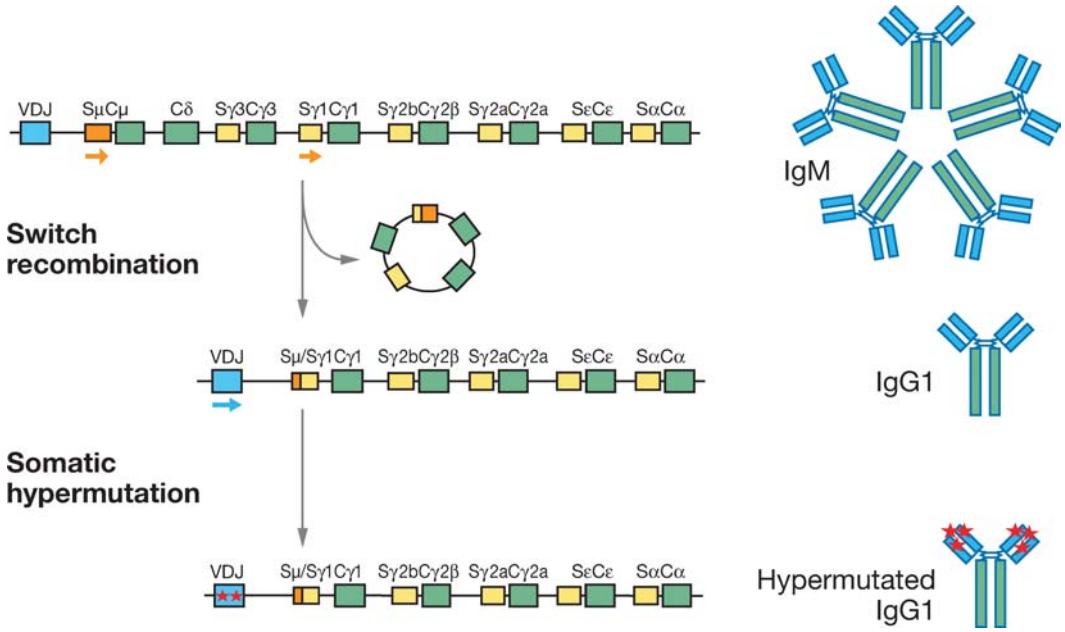

Figure 2

Class switch recombination and somatic hypermutation at the murine heavy chain locus. Switch recombination joins a rearranged and expressed heavy chain variable region (VDJ) to a new downstream constant region (C), forming junctions within switch (S) regions. Somatic hypermutation alters V region sequence (*stars*). V and S regions are activated for diversification by transcription (*colored arrows*) and is accompanied by hypermutation (*right*). The figure illustrates recombination from Sμ to Sγ1, which results in a switch from IgM to IgG1 antibody expression; changes in Ig molecules are shown at the right.

A Shared Pathway Produces the Distinct Signature Outcomes of Ig Gene Diversification

That the pathways of Ig gene diversification are mechanistically related was first suggested by absence of a clear evolutionary pattern to the use of templated or nontemplated mutation. Nontemplated mutation alters Ig V regions in humans and mice (61, 64, 83), but templated mutation alters V regions in closely related mammals, including common farm animals such as sheep, cattle, and pigs (16, 57, 72), as well as chicken and other fowl (70); and both templated and nontemplated mutation can occur in a single species, most notably rabbit (57). This led to the proposal that targeted mutagenesis in all organisms is initiated by a DNA lesion leading to a break, which could then be processed by an error-prone repair pathway, to produce nontemplated mutation; or by recombinational repair to produce templated mutation (63). This model has been borne out by evidence that, in chicken cells, impaired homologous recombination due to deficiencies in RAD51 paralogs (114) or BRCA2 (43) causes a shift from templated to nontemplated mutation. Gene conversion, somatic hypermutation, and switch recombination were united within a single diversification pathway by experiments showing that all three processes depend upon a single B cell–specific enzyme, AID (2, 42, 76, 109).

AID DEAMINATES DNA TO INITIATE Ig GENE DIVERSIFICATION

AID was first identified by Honjo and collaborators in a screen for genes induced specifically upon activation of mammalian B cells

(77). Targeted deletion of the murine *Aid* gene was then shown to essentially abolish both somatic hypermutation and class switch recombination in activated murine B cells (76). In humans, AID is the product of *HIGM2*, a well-characterized locus associated with severe immunodeficiency characterized by lymph node hyperplasia, the absence of somatic hypermutation, and impaired switch recombination evident as low levels of serum antibodies of IgG, IgA, and IgE classes, and elevated levels of IgM antibodies (109). Elevated IgM levels often are evident as one physiological consequence of impaired switch recombination, reflecting an attempt by the immune system to compensate for the deficiencies in other Ig isotypes.

AID is Expressed in Germinal Center B Cells

AID expression is restricted to brief windows in B cell development during which Ig gene diversification occurs. In chicken, sheep, and rabbit, diversification of the limited combinatorial repertoire is especially active in young animals. In newly hatched chicks, diversification occurs in a specialized lymphoid organ, the bursa, which has no homolog in mammals (the term "B" lymphocyte originally distinguished "bursa-derived" lymphocytes from "thymus-derived" or "T lymphocytes" in the chicken); and AID expression is coordinated with bursal diversification (136). In humans and mice, AID is induced in antigen-activated B cells (77, 90). These cells populate germinal centers, histologically distinctive microenvironments within spleen, lymph nodes, tonsils, appendix, and intestinal Peyer's patches, where B cells switch, hypermutate, and undergo selection for high-affinity clones. BCL6 is required for germinal center formation, and in germinal center B cells, BCL6 downregulates p53, thereby creating a permissive environment for DNA diversification (96).

A chicken bursal lymphoma line, DT40, carries out constitutive Ig gene conversion; and hypermutation is ongoing in a considerable subset of human B cell lines derived from germinal center tumors. These lines have proven valuable models for studies of Ig gene diversification.

AID Overrides Uracil DNA Repair to Mutate C → U in *Escherichia coli*

AID is related to APOBEC1 (77), a cytidine deaminase that edits a specific cytidine in the apolipoprotein B transcript to produce a nonsense codon that results in expression of a truncated polypeptide. This initially suggested that AID might target a specific mRNA encoding a master regulator in the form of a critical nuclease or transcription factor, rather than participate directly in the diversification mechanism. Uracil in DNA is a frequent and spontaneous lesion, which can originate by deamination of C → U at C/G base pairs, or by incorporation of dUTP opposite A during replication (6, 52). It is repaired by an efficient base excision repair pathway, in which uracil nucleoside glycosylases remove the uracil base, abasic endonucleases nick the phosphodiester backbone at the abasic site, DNA polymerase β synthesizes new DNA, and ligases seal the nick, as shown in **Figure 3** (6, 52).

The hypothesis that AID directly deaminates DNA in living cells was tested by asking if expression of AID in *E. coli* stimulates mutation of reporter genes (94). Cytidine deamination at a C/G base pair will produce a U•G mismatch, and upon replication a C/G → T/A transition mutation will be fixed in genomic DNA. Consistent with AID-initiated C → U deamination as the source of mutations, reporter genes carried a high proportion of transition mutations at C/G pairs, and mutations levels increased in a *ung-* strain, deficient in the major uracil nucleoside glycosylase activity (94). Thus, AID expression can overwhelm the normally efficient uracil DNA repair pathway to cause mutagenesis in *E. coli*.

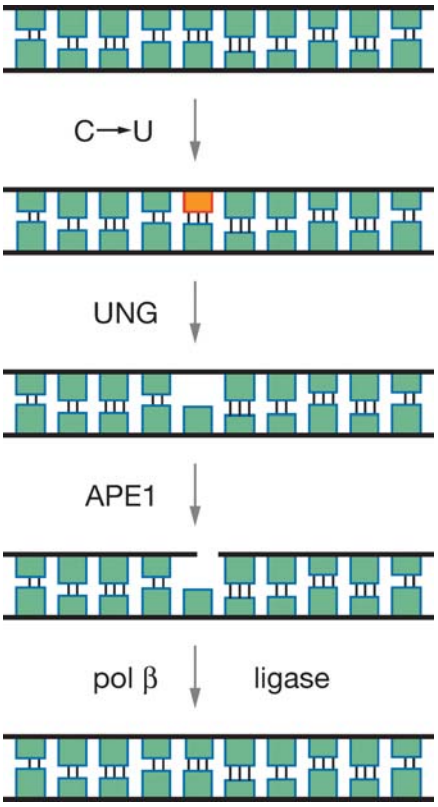

Figure 3

Faithful repair of uracil in DNA. Uracil is repaired by a conserved and ubiquitous pathway: uracil nucleoside glycosylase (UNG) removes the uracil base (*orange*), AP endonuclease 1 (APE1) cleaves the phosphodiester backbone at the abasic site, and DNA polymerase β and ligases repair the gap.

AID Deaminates Single-Stranded DNA: Transcriptional Targeting of Diversification

Biochemical analysis provided a second line of evidence that AID participates in the mutagenic mechanism. Recombinant AID had been shown to deaminate cytidine in vitro (77). Nonetheless, the demonstration that AID deaminates C → U in single-stranded DNA, within an artificial transcription bubble, or within a transcribed region, but not in duplex DNA (13, 21, 28, 103, 123) provided a breakthrough in understanding, immediately explaining why transcription is critical for Ig gene diversification: Transient denaturation accompanying transcription produces the single-stranded DNA substrate for AID.

Recombinant AID displays a 7- to 15-fold preference for deamination of Cs in the nontemplate DNA strand in vitro (12, 21, 95, 103). This was initially puzzling. There is no clear strand bias in products of hypermutation (73), so AID almost certainly deaminates both DNA strands. Two tactics may provide access to the template strand. Interaction with the eukaryotic single-strand binding factor, RPA, facilitiates deamination of the nontemplate strand in transcribed substrates in vitro (20). AID will also deaminate both strands within regions of negative DNA supercoiling (120), which could contribute to targeting AID to both DNA strands in the region ahead of an advancing RNA polymerase.

Hypermutation Hotspots are Deamination Hotspots

AID preferentially deaminates C's within the sequence context WRC (W = A/T; R = purine) both in vitro and in vivo (12, 95). Analysis of sequences of vast numbers of hypermutated mammalian V regions had shown that a significant fraction of mutations occurred within motifs conforming to the consensus WRCY (complement, RGYW; Y = pyrimidine) (111), which includes the preferential motif for AID deamination. Thus, hypermutation hotspots are deamination hotspots.

Hypermutation hotspots were initially interpreted to reflect selection for high-affinity antibodies because they appeared to cluster within the complementarity-determining regions (CDRs), which make direct contact with antigen. The discovery that they reflect substrate preference of AID explains why the same hotspot appears in antigen-selected V regions, unselected V regions, and in non-Ig reporter genes (47). Moreover, V regions may have evolved to take advantage of AID-induced hypermutation, as motifs for AID deamination concentrate within the CDRs,

where mutation can fine-tune antigen recognition; and are depleted within framework regions, where mutation can destroy antibody function. Further suggestive of coevolution of V regions and AID, each of the two serine codons, AGY and TCN, is favored in different subdomains of the V regions: AGY, which more closely approximates the deamination consensus, is favored in CDRs; and TCN in framework regions (47). This localization of AGY serine codons in the CDRs extends back to pufferfish, which encode an AID homolog with a preferred deamination motif the same as that of mammalian AID (9, 22).

PARADOXICAL MUTAGENIC FUNCTIONS FOR UBIQUITOUS REPAIR FACTORS

The evidence that AID deaminates DNA directly provided a mechanism for the first step in mutagenesis, but raised a new question: How is repair of uracil in DNA diverted from faithful to mutagenic pathways at B cell Ig genes? Ectopic expression of AID is sufficient to induce switch recombination (85) or somatic hypermutation (139) in engineered minigenes in mammalian fibroblasts, so downstream steps in the AID-initiated mutagenic pathway must therefore be carried out by ubiquitous factors. Indeed, the factors shown to participate in this pathway are highly conserved repair factors that are normally essential for maintenance of genomic stability. Paradoxically, they create irreversible changes in genomic sequence and structure in the process of repairing AID-initiated DNA damage at B cell Ig genes.

UNG Promotes Ig Gene Diversification

Neuberger and collaborators reasoned that if UNG (uracil nucleoside glycosylase) attacks U produced by AID deamination, then diminished levels of UNG would affect Ig gene diversification. This hypothesis was tested in an *XRCC2−/−* derivative of the chicken B cell line, DT40, which accumulates both nontemplated and templated mutations at the Ig loci (114). Consistent with function of UNG in the mutagenic pathway, decreased levels of UNG activity in DT40 *XRCC2−/−* mutants resulted in an increase in transition mutations at C/G pairs (25). Moreover, in *Ung−/−* mice, switch recombination is impaired, and there is increase in transition mutations at C/G pairs (101). Transitions at C/G pairs can occur when a U•G mismatch generated upon deamination is fixed upon replication (**Figure 4**). In contrast to these results, one recent report claimed that class switch recombination does not depend upon UNG deglycosylase activity (10). However, the deglycosylase activity of the mutants tested in those experiments appears not to have been completely impaired (124), which calls into question the conclusions of that other report.

Analysis of human genetic disease provided additional support for function of UNG in the AID-initiated pathway of targeted diversification (44). A severe human immunodeficiency, HIGM ("Hyper IgM"), characterized by elevated IgM and low or absent serum IgG and IgA, results from mutations in the *UNG* gene (44). As in *Ung-/-* mice (101), in this human genetic disease deficient switch recombination is accompanied by a shift in the hypermutation spectrum, increasing the fraction of transition mutations at C/G base pairs. Notably, the absence of UNG activity in affected individuals had not been detected as a DNA repair deficiency before it was revealed as an immunodeficiency (44).

What is the fate of the abasic site created by UNG? Cells contain numerous enzymes that can cleave DNA containing abasic sites, including abasic endonucleases like APE1, which cleave the phosphodiester backbone; and lyases, which attack the deoxyribose 1′ C (6, 52). The cleaved DNA could then participate directly in gene conversion or class switch recombination (**Figure 4**). Alternatively, low-fidelity repair at the cleaved end or opposite the abasic site ("translesion" synthesis) could result in point mutations.

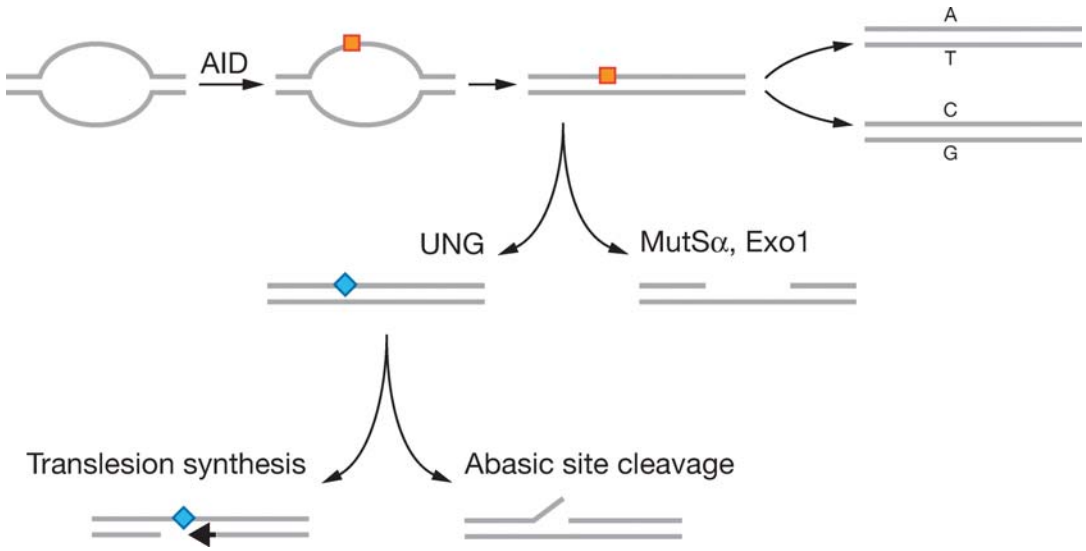

Figure 4

Mutagenic outcomes of AID-initiated deamination. AID deaminates C → U (*orange square*) in transcribed DNA (represented as a bubble to emphasize the transcriptional requirement for deamination). The duplex containing the U•G mismatch has three possible fates: replication will fix C/G → T/A transition mutation (*top right*); resection by MutSα-Exo1 will reveal a patch or gap (*center right*); and deglycosylation by UNG will create an abasic site (*blue diamond*) which undergoes further mutagenic repair (*center*). Mutations may be introduced opposite the abasic site by low-fidelity DNA synthesis (translesion synthesis, *bottom left*); or an abasic endonuclease or lyase may cleave and generate a DNA break (abasic site cleavage, *bottom right*).

NBS1 and the MRE11/RAD50/NBS1 Complex in Ig Gene Diversification

The conserved MRE11/RAD50/NBS1 (MRN) complex is involved in the response to DNA damage and cell-cycle checkpoint control. NBS1 is the regulatory subunit, which responds to and regulates ATM kinase and governs nuclear localization and activities of the MRE11/RAD50 nuclease. NBS1 displays AID-dependent localization to S regions in activated B cells (93), and conditional knockout of *Nbs1* in murine B cells results in impaired switch recombination (51, 108). As a member of the MRN complex, NBS1 promotes both somatic hypermutation in human cells and gene conversion in chicken cells (138). MRN is also necessary for recombination-associated mutagenesis within Sμ and at switch junctions, as the spectra of junction mutations are altered in B cells from individuals with Nijmegen break syndrome, due to deficient NBS1, and AT-like disease, resulting from deficient MRE11 (56); and junctional mutagenesis is absent in ataxia telangiectasia (AT), due to deficient ATM (86, 87).

MutSα (MSH2/MSH6) and Exo1 Promote Switching and Hypermutation

In single-base mismatch repair, MutSα (the MSH2/MSH6 heterodimer) recognizes the mismatch, recruits MutLα (MLH1/PMS2), which recruits and promotes DNA excision by ExoI, to generate a gap, which undergoes high-fidelity repair and religation (41, 53, 115, 125). One might therefore have predicted that mismatch repair factors would correct AID-induced mutations and prevent mutagenesis.

Counterintuitively, hypermutation and switch recombination decrease upon targeted disruption of *Msh2*, *Msh6*, and *Exo1* in mice AID (5, 32, 60, 68, 100). In wild-type mice, hypermutated sites include both C/G pairs and also T/A pairs, but in the absence of MutSα or Exo1, mutations focus at C/G pairs (45, 97, 133) and at sequences conforming to the AID deamination motif, WRCY (100). Switch junctions similarly focus at the WRCY motif (32, 117, 131), a deamination consensus. Mutation of the *Msh2* ATPase, which abolishes function in mismatch repair, recapitulates changes in hypermutation spectrum evident in *Msh2−/−* mice, but has little effect on switch recombination (67). Deficiencies in *Pms2* and *Mlh1* (MutLα) alter levels or spectra of hypermutation (17, 48, 49, 135), and affect levels of switching only modestly but cause an increased dependence on microhomology-mediated end joining (33, 119).

Taken together, these results identify key functions in hypermutation and switch recombination for MutSα and Exo1, and suggest more subtle roles for other mismatch repair factors. Purified recombinant hMutSα binds specifically to duplex DNA carrying U•G mismatches (58, 134), suggesting that mismatch repair factors may collaborate to resect the region containing U (**Figure 4**). Resection will reveal a patch of DNA that could be the template for low-fidelity DNA synthesis, and generate ends that could participate in gene conversion or switch recombination.

UNG and *MSH2* Define Two Paths to Mutagenesis

The differences between mutation spectra in wild-type and MutSα-deficient mice suggested that there might be two distinct phases of hypermutation, the first MutSα-independent and focused at hotspots; and the second MutSα-dependent, and more evenly distributed (100). This proposal was validated by studying Ig gene diversification in *Msh2−/−Ung−/−* mice (99). In the double mutant there is no switch recombination and no hypermutation at T/A pairs, and all somatic hypermutation consists of transition mutations at C/G pairs, apparently produced upon replication of U•G mismatches created by AID. Thus, *Ung* and *Msh2* are defining elements in two pathways for mutagenic repair of AID-initiated damage. The ability of MutSα to recognize U•G mismatches in vitro (58, 134) suggests that MutSα and UNG may compete for repair of uracil at Ig genes (**Figure 4**).

S REGIONS ARE ACTIVE PARTNERS IN SWITCH RECOMBINATION

We tend to think of proteins acting on DNA substrates. However, the transcriptional requirement for Ig gene diversification suggests a more active role, and structures formed within transcriptionally activated S regions prove to be specific targets for recombination factors.

S Regions: A Mix of G-Rich Repeats and Deamination Hotspots

The active role of the S regions in switching is shown by the observation that frequencies of switch recombination correlate with S region target length (145). S regions carry two characteristic sequence motifs, G-rich degenerate repeats, and hotspot motifs for AID deamination. These are distributed differently in different S regions. An extreme case is a Xenopus switch region, which is AT-rich and contains reiterations of the tetramer AGCT motif (78). This sequence conforms to the AID deamination consensus, WRC, and can substitute effectively for a murine S region by providing a dense array of sites for AID (144). Similarly, the murine Sμ region contains an extended GAGTC repeat. Deletion of the GAGTC repeat to leave mainly G-rich repeats reduces the efficiency of switching somewhat, and makes recombination dependent on *Msh2* (74).

G-Loops in Transcribed S Regions are Deaminated by AID and Synapsed by MutSα

S regions are G-rich on the nontemplate (top) strand, and must be transcribed in *cis* and in the correct physiological orientation to support recombination (122). Switch region transcription causes formation of characteristic structures that have been identified in electron microscopic images as extended G-loops (30). G-loops contain a cotranscriptional RNA:DNA hybrid on the template strand, as anticipated by gel electrophoresis (23, 104, 105, 128, 140) and atomic force microscopy (75); and regions of G4 DNA interspersed with single-stranded DNA on the G-rich strand (30). Two nucleases active in transcription-coupled repair, ERCC1/XPF and XPG, can cleave the upstream and downstream junctions between the RNA:DNA hybrid and duplex DNA in transcribed S regions in vitro (128). However, analyses of murine models revealed no effect on switch recombination resulting from mutation of *Xpf* (130) or *Xpg* (129), and at most a modest effect of deletion of *Ercc1* (118).

Two factors shown by genetic analysis to be key to switch recombination do recognize G-loops in transcribed switch regions. AID interacts specifically with and deaminates the G-rich strand of G-loops (31, 141), consistent with its preference for single-stranded DNA. MutSα binds to G4 DNA with affinity considerably higher than for heteroduplex (T•G or U•G) mismatches; and MutSα bound to G-loops promotes DNA synapsis between transcriptionally activated S regions (58). The RNA:DNA hybrid within a G-loop may prolong DNA denaturation, thereby increasing accessibility of the DNA to AID or MutSα.

Many Factors Collaborate in Switch Recombination

Switch recombination depends upon four factors involved in the DNA damage response: the cell cycle regulator ATM (62, 106); the phosphorylated variant histone γ-H2AX (107); NBS1, the regulatory component of the MRE11/RAD50/NBS1 (MRN) complex (51, 108); and the p53-interacting protein, 53BP1 (65, 132). ATM and γ-H2AX are required only for inter-S region recombination and not for intra-S region recombination, leading to the suggestion that they facilitate synapsis by promoting higher-order chromatin remodeling (106, 107). Switch recombination rejoins the chromosome and generates switch circles carrying the excised fragment (19, 61, 66), as shown in **Figure 2**. It is therefore not surprising that switch recombination also depends upon factors involved in nonhomologous end-joining, including Ku and DNA-PKcs (113). Interactions with these or other factors may explain the observation that switch recombination depends upon a 10 amino acid region at the very C terminus of AID, a region unnecessary for cytidine deamination or for gene conversion or somatic hypermutation (7, 121, 126).

DNA BREAKS AND DIVERSIFYING Ig GENES

Figure 5 presents a model for how DNA breaks produced UNG-dependent abasic site cleavage or MutSα-Exo1-dependent resection can be processed to produce the three signature outcomes of Ig gene diversification, by pathways that combine elements of error-prone DNA synthesis, homologous recombination, and non-homologous end-joining.

Error-Prone Polymerases and Point Mutations

Error-prone polymerases are a likely source of nontemplated mutations that accompany gene conversion, hypermutation, and switch recombination. Among the error-prone polymerases characterized in recent years (36, 38, 54), the strongest candidate for function in hypermutation is pol η, a Y-family

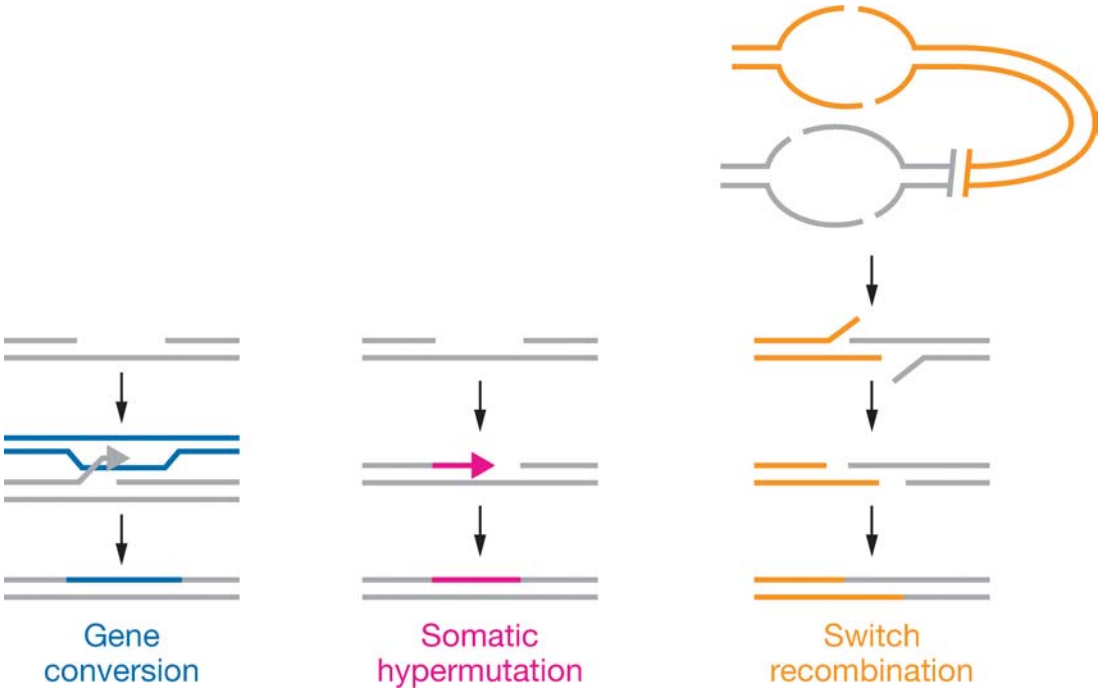

Figure 5

DNA breaks give rise to the signature products of Ig gene diversification. *Left*, a single break in the nontemplate DNA strand of a V region initiates gene conversion (*blue*) with the appropriate 5′ to 3′ directionality. *Center*, error-prone repair using a DNA end as primer creates nontemplated mutations at a V region, leading to hypermutation (*pink*). For other paths to hypermutation, see text and **Figure 4**. *Right*, two cleaved and synapsing switch regions (*gray and orange*) undergo microhomology-mediated nonhomologous end-joining.

polymerase encoded by the *XPV* gene. Mutations in *XPV* result in xeroderma pigmentosum variant disease, characterized by faulty repair of UV damage. In individuals deficient in pol η, hypermutation at T/A pairs is diminished without apparent immunodeficiency (35, 146, 147). The pol η mutagenesis spectrum in vitro supports possible function as an T/A mutator in somatic hypermutation (92, 112). Moreover, MutSα, which is necessary for hypermutation at T/A pairs (82, 99), stimulates pol η activity in vitro (134). Another Y-family polymerase, pol ι, appeared to be a strong contender as a G/C mutator (24, 34, 98), but enthusiasm dampened with the discovery that strain 129 mice naturally lack pol ι but hypermutation is not altered (71). Pol ζ, a B-family polymerase, can extend from a mismatched primer and may work in collaboration with other mutagenic polymerases (27, 46, 81, 142). Error-prone polymerases interact with one another and the replication apparatus, which may facilitate redundancy and make it difficult to establish critical function of any single polymerase.

Nontemplated base changes in hypermutation have been assumed to originate by misincorporation by low-fidelity polymerases, using the four canonical deoxynucleoside triphosphates. An intriguing alternative hypothesis is that mutation results from incorporation of nonstandard bases, such as dUTP, and subsequent repair by either high- or low-fidelity polymerases (82).

A Break is Critical for Gene Conversion

A simple model for gene conversion is that AID and UNG together create an abasic site, which is cleaved; and the resulting 3′ end undergoes homologous repair, promoted by RAD51 paralogs and BRCA2 (43, 114), copying new sequence information from a donor pseudogene (**Figure 5**, left). The documented 5′ to 3′ directionality of gene conversion (69) suggests that a single break in the top strand creates the primer for gene conversion. There has as yet been no genetic test of function of MutSα or Exo1 in Ig gene conversion, although these factors could in principle provide a 3′ end for strand invasion.

Gene conversion at chicken Ig genes produces not only templated but also nontemplated mutations, like those generated by somatic hypermutation (1). Moreover, the balance shifts to nontemplated point mutations as a result of inhibition of UNG (26); or interference with homologous recombination by mutation of *RAD51* paralogs or *BRCA2* (43, 114) or deletion of pseudogene sequence donors (3). Thus, the DNA end that is the primer for gene conversion (**Figure 5**, left) can alternatively undergo repair by hypermutation (**Figure 5**, center).

Breaks May Produce Somatic Hypermutation

The U•G mismatches introduced by AID can be processed in three ways leading to nontemplated mutations. Repair by an error-prone polymerase at a break (**Figure 5**, center) would produce nontemplated muation, as is found in hypermutation. Two other routes to mutagenesis do not involve a DNA break: replication to generate C/G → T/A transition mutations—the only mutations observed in *Msh2-/-Ung-/-* double knockouts (99); and mutagenic synthesis opposite an abasic site produced by UNG (**Figure 4**). Breaks almost certainly accompany hypermutation in vivo, as unselected hypermutated V regions contain short DNA deletions, like those found in S regions (39).

Switch Recombination Requires Two DNA Ends

Switch recombination joins two S regions (**Figure 5**, right), deleting a large stretch of chromosomal DNA. Switching requires breaks in both DNA strands, which could arise from UNG or Mutsα-Exo1-dependent cleavage of both strands, or by replication at a single-strand break to create a blunt or staggered end. Switching, like V(D)J recombination, depends on nonhomologous end-joining factors (113) to process DNA ends. Following switch recombination, the excised S regions can be recovered as nonreplicating circles (**Figure 2**). In essentially all processes of programmed recombination that involve free DNA ends, the ends are sequestered until recombination is completed. This suggests that switch circles may be products of reciprocal recombination, a question that could almost certainly be addressed using single-cell PCR to compare junctions in switch circles and chromosomal DNA.

Once a B cell has completed switching, switch regions resemble products of hypermutation: Sμ switch regions accumulate AID-dependent point mutations (79), and switch junctions are flanked by recombination-associated point mutations (29, 116). Whether the mechanism of S region mutagenesis is entirely or only partially shared with that of hypermutation is not yet resolved.

Single-Strand Breaks, Not Double-Strand Breaks, in AID-Initiated Diversification

Experiments seeking to define DNA intermediates of Ig gene diversification have identified double-strand breaks in mammalian B cells

(14, 18, 88, 143). However, these breaks appear not to depend upon ongoing hypermutation (14) or AID expression (15, 89, 143), and may not be relevant to AID-initiated diversification. Single-strand DNA breaks (4, 50) and staggered ends (143) have been identified, and are consistent with current understanding of the cleavage mechanism. Staggered ends could result from independent cleavage of both DNA strands, or replication of a region containing a nick or gap on one strand, which generates one double-strand break and one staggered end.

TARGETING AND MISTARGETING OF GENE DIVERSIFICATION

How is Diversification Targeted to Ig Genes?

AID is targeted (almost) exclusively to the expressed Ig genes. The requirement for a single-stranded substrate produced upon transcriptional activation provides one key to AID targeting. Targeting does not depend upon specific *cis*-elements or chromosomal position, but may depend on modifications in chromatin or specific interactions of AID with RNA polymerase II (59, 80, 137). Key open questions are how actively transcribing non-Ig genes are protected from deamination, and how deamination is limited to a few kb downstream of the promoters, protecting the C region from mutagenesis.

Mistargeting: Hypermutation and Translocation of Proto-Oncogenes

The c-*MYC* proto-oncogene undergoes both aberrant hypermutation and translocation in B cell tumors (91). This genetic instability reflects targeting of AID to c-*MYC*. c-*MYC* is transcribed in activated B cells, making it a potential substrate for deamination by AID. Moreover, AID is required for c-*Myc* translocation leading to tumorigenesis in a murine model for Burkitt's lymphoma (102). Electron micrographic imaging reveals that G-loops form within the transcribed c-*MYC* gene, with structures similar to those that form in transcribed S regions (30, 58), and that these G-loops are targets for AID (31). G-loops map to a G-rich region within the first exon and intron of the transcribed c-*MYC* gene, the same zone that undergoes aberrant hypermutation and translocations. In addition to c-*MYC*, a small subset of other non-Ig genes undergo aberrant hypermutation or translocation in B cells, including the *BCL6*, *CD95/FAS*, *RHO/TTF*, *PAX-5*, and *PIM1* proto-oncogenes (55, 91); and the B29 and MB1 B cell receptor genes (40). Further analysis of mistargeting of AID to these genes may provide insights into how AID is targeted with considerable accuracy to the Ig loci.

AID is But One Member of the APOBEC Gene Family

AID has some interesting relatives in the APOBEC family of cytidine deaminases, which may function as effectors of innate immunity and as sources of somatic mutagenesis in non-B cells. Two of these, APOBEC3F and APOBEC3G, prevent retroviral replication and deaminate retroviral cDNAs during second-strand synthesis (11), although the antiviral effects may not depend entirely upon deamination (84). Each APOBEC has a distinctive, context-dependent mutation pattern (9), raising the interesting possiblity that viral targets for specific APOBECs may have coevolved, like V regions and AID. Some APOBECs have a wider expression profile than AID, which is restricted to activated B cells (9, 22), suggestive of very general functions. An expansion of APOBEC3-related genes has occurred during human evolution (9, 22), creating a handful of related deaminases—orphan enzymes, the targets of which are yet to be discovered.

SUMMARY POINTS

1. Ig gene diversification is essential to the immune response and produces three signature outcomes: gene conversion (templated mutation), somatic hypermutation (nontemplated mutation) and class switch recombination (region-specific DNA deletion).
2. AID initiates Ig gene diversification by deaminating cytidines in transcribed Ig genes at consensus motifs, identical to hypermutation hotspots. AID deaminates single-stranded DNA but not double-stranded DNA, explaining how deamination is targeted to transcribed genes, but not how transcribed non-Ig genes escape deamination.
3. Conserved factors UNG, MRN, MutSα, and Exo1 play paradoxical roles in Ig gene diversification, promoting—rather than preventing—genetic instability.
4. Targeting of diversification depends upon specificity of AID for single-stranded DNA, which restricts deamination to transcribed genes; and formation of characteristic structures, G-loops, within transcribed S regions. How many transcribed regions escape attack by AID is not understood. Proto-oncogenes like c-*MYC* are mistargeted, which can lead to aberrant hypermutation, translocation, and tumorigenesis in B cells.

PERSPECTIVE AND FUTURE DIRECTIONS

In the past few years Ig gene diversification has been pried out of the metaphorical black box and defined in genetic and biochemical terms. We have learned that a single, simple DNA modification, cytidine deamination, can drive three processes that had once seemed unrelated: hypermutation, gene conversion, and class switch recombination. We have seen how these specialized processes relate to ubiquitous and conserved pathways of DNA repair and recombination that maintain genomic stability in all organisms, and we have been forced to appreciate that factors which normally promote genomic integrity can be active participants in its opposite. Some future directions are clear. We need to learn more about targeting of deamination and functions of deaminases in human biology. We need to learn more about how Ig gene diversification occurs, not only at the molecular level but also within the larger context of chromatin structure and nuclear organization. These details of immune diversification will have implications for our broader understanding of biology. Most important, if recent progress has shown us anything, it is to expect surprises. As we pose questions for the future, we can look forward to answers that go beyond our ability to hypothesize and speculate.

ACKNOWLEDGMENTS

I thank members of my laboratory and my colleagues for valuable discussions, and the NIH for supporting our research on Ig gene diversification (GM39799, GM41712).

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