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The regulation of somatic hypermutation

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Somatic hypermutation and class switch recombination cause genetic alterations in immunoglobulin (Ig) genes, which underlie the generation of the secondary antibody repertoire in B lymphocytes. Both processes require activation-induced cytidine deaminase (AID), whose mechanism of action in not yet known in detail, but which mediates the accumulation of point mutations in the Ig locus. This highly mutagenic process must be tightly controlled, and multiple levels of regulation might exist. Recent experiments show that AID deaminates deoxycytidine to deoxyuridine in single-stranded DNA. This mutagenic event is targeted to actively transcribed sequences, and the specificity of deamination might be related to the chromatin structure of the transcription complex.

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Abbreviations

AID	activation-induced cytidine deaminase
CSR	class-switch recombination
dC	deoxycytidine
dsDNA	double-stranded DNA
dU	deoxyuridine
Ig	immunoglobulin
SHM	somatic hypermutation
ssDNA	single-stranded DNA
UDG	uracil–DNA glycosylase

Introduction

B lymphocytes can produce antibodies to almost any foreign substance. At the molecular level, diversity is initially generated by V(D)J recombination, a process that combines and assembles antigen receptor genes from a pool of gene segments. After V(D)J recombination, B lymphocytes leave the bone marrow and undergo two further receptor diversification processes: somatic hypermutation (SHM), which is initiated by the binding of antigen and introduces point mutations into the variable region of immunoglobulin genes (a region that codes for the antigen-binding pocket of antibody molecules); and class-switch recombination (CSR), which interchanges the constant region of antibodies to produce molecules with the same specificity but different effector functions [1]. Though SHM and CSR were initially thought to employ quite distinct molecular mechanisms, a recent breakthrough has shown that both require the newly discovered gene *aid*, which encodes activation-induced cytidine deaminase (AID or AICDA).

AID protein expression is thought to be largely restricted to developing germinal centers where SHM and CSR occur [2]. AID-deficient mice and humans develop normally and respond to challenge with antigens but they fail to hypermutate or undergo CSR [3,4]. AID was initially observed to have homology to APOBEC1, the prototype cytidine deaminase, which functions as an RNA-editing enzyme by deaminating deoxycytidine (dC) to create deoxyuridine (dU) [2]. Such evolutionary considerations led the discoverers of the enzyme to suggest that AID might also edit RNA, probably an mRNA molecule ubiquitously present in vertebrate cells, whose editing would create an enzyme crucial for both SHM and CSR. Yet there is no logical reason why AID could not deaminate dC to dU. If this were the case, AID would create mutations of dC or the complementary base-paired deoxyguanosine (dG) residues, which is in fact the bias of the hypermutation machinery. There are two lines of evidence to suggest that AID deaminates DNA. First, overexpression of AID leads to hypermutation in mammalian as well as bacterial systems [5•–7•]; it is hard to imagine AID editing the same RNA in mammalian cells as in bacteria. Second, if AID were to create dU in the genome such residues would eventually need to be removed and the DNA repaired. Uracil–DNA glycosylase (UDG or ung) is one of the main enzymes that removes uracil from the genome, and in its absence SHM is, in fact, affected and CSR is severely impaired [8]. This places AID and UDG/ung in the same pathway, and strongly suggests that UDG/ung removes from the genome the dU created by AID through dC deamination. Thus, the theory that AID deaminates and edits RNA has been supplanted by the widely accepted model of an enzyme that targets DNA and, in the process, creates highly mutagenic lesions.

Obviously, such a mutator would have to be stringently regulated in the cell. Multiple levels of regulation may be imagined but, in this review, we will focus on the following three: regulation of AID expression and localization; AID-dependent DNA deamination and the regulation of the SHM reaction in the context of transcription; and, higher order structures that regulate AID-mediated SHM *in vivo* (including enhancers and other targeting elements).

Regulation of AID expression and localization

Multiple pathways lead to AID expression, including signals that induce CSR in naïve B cells (such as lipopolysaccharide [LPS], IL-4, CD40 ligation and TGF-β), and signals that induce SHM in human lymphoma lines (such as IgM–CD19–CD21 cross-linking). Yet very little is known about the set of common factors that converge onto the AID gene as a result of these diverse stimuli. Recently, Sayegh and colleagues [9•] have shown that the products of the E2A gene, E2-2 and E47, induce transcription of the AID gene, both in a B-cell line and in splenic B cells activated *in vitro*, by binding two E-box sites within the first intron of the AID gene. This finding may explain why inhibition of E2A binding interferes with CSR [10]. Because expression of AID is restricted to B cells, other factors should also be involved in its regulation. Gonda and colleagues [11•] suggest that Pax5 and the inhibitor of E2A binding Id2 are two such factors: in their experiments the forced expression of Id2 reduced AID expression and inhibited CSR, whereas forced expression of Pax5 induced AID gene expression in pro-B-like cell lines. Therefore, the balance between Pax5 and E-box-binding proteins plays an important role in the regulation of AID expression.

Whatever the regulation of its expression, when AID protein is made it must travel to the nucleus where it deaminates DNA. There is ample evidence, however, that AID is found predominantly in the cytoplasm [12•], and it is possible that it only enters the nucleus to inflict damage under very controlled conditions, perhaps when complexed with shuttle proteins. Reynaud and colleagues [13•] have shown that, when AID is overexpressed in a human lymphoma line, it is indiscriminately mutagenic until the cells are specifically induced to hypermutate, at which point the mutagenic activity of AID becomes restricted to the variable region loci.

Regulation of AID-mediated DNA deamination: lessons from *in vitro* studies

In the nucleus AID must find its target locus. A hint about how this might be accomplished comes from recent biochemical experiments. Several groups have shown that AID binds [14•] and deaminates only single-stranded DNA (ssDNA; [14•,15,16•]). ssDNA is found in the cell during replication and transcription, and two laboratories have shown that, in *Escherichia coli*, AID preferentially deaminates the non-transcribed DNA strand that is exposed during transcriptional elongation [17•,18]. An intriguing possibility is that AID may be targeted to the ssDNA exposed by the transcription complex by binding nascent RNA [17•] or the RNA–DNA hybrid [16•]. Recent work from Goodman and co-workers [15] suggests that recombinant AID is, in fact, activated after treatment with RNaseA. However, this is only true for the recombinant version of the protein used in [15], and at least two other groups have reported that RNaseA does not modulate AID activity [14•,18].

If AID is targeted to DNA by the transcription complex, then *in vitro* transcription of double-stranded DNA (dsDNA) in the presence of AID should lead to deamination of the transient ssDNA template. Indeed, Goodman and colleagues [19] have recently reported that AID can deaminate dsDNA transcribed by T7 polymerase. Others, however, have reported the opposite result, that transcription by T7 polymerase in the presence of AID does not lead to DNA deamination unless the sequence is purine rich and able to form an RNA–DNA hybrid (an R-loop) [16•]. In that case, *in vitro* transcription would simply serve to generate the structure that AID would target *in vivo*.

In vivo dependence of mutation on transcription

The link of SHM with transcription is amply supported by the *in vivo* literature. It has long been known that the variable region promoter forms an upstream boundary to hypermutation [20], that mutations peak over the rearranged variable region, and that the rate of mutation over a random sequence varies depending on its distance from the variable region promoter [21]. When the variable region resides within its natural distance from the promoter, hypermutation declines precipitously past the rearranged VJ segment [22]. Furthermore, when the promoter is removed by gene targeting, hypermutability of the locus is vastly diminished [23], whereas when the promoter is duplicated upstream of the constant region that region is induced to hypermutate [24]. Finally, the rates of hypermutation are proportional to the rates of transcription [25,26]. Therefore, the link between AID-mediated hypermutation and transcription seems to be well established.

Sequence-specific targeting of mutation

AID may be targeted to transcribed immunoglobulin (Ig) genes, but it is quite clearly not targeted to all transcribed genes. This could be explained if AID was expressed at low and possibly limiting levels, so that there might not be enough enzyme to mutate all transcribed genes. This is probably not the case: overexpression of AID leads to hypermutation in all organisms tested, but despite high protein levels, the rates of mutation are fairly low. So, what recruits AID preferentially to some genes but not others? Because mutations do not seem to be absolutely sequence dependent, Gearhart and others [27] first postulated a possible role for DNA secondary structure as a recognition motif, but mutations did not necessarily coincide with particular secondary structure elements. Using computer predictions, Storb and colleagues [28] recently suggested a role for RNA secondary structure and proposed that, when the V region is transcribed, folding of the nascent mRNA could induce pausing. An exhaustive study by the same investigators, however, found that

regions of strong secondary structure (either DNA or RNA) are not inherently highly mutable [29•]. In addition, no clear relationship between known RNA polymerase II pause sites and mutations was observed in a human c-myc gene translocated into the Ig locus [30]. Yet, experiments in which transcriptional elongation is pharmacologically hindered by the intercalation of actinomycinD (a situation in which ssDNA would be abundantly exposed by the arrested polymerase) the rates of hypermutation remain unchanged, although what happens to the patterns of mutation is not as clear [13•]. Thus, it is still possible that stalling or transient pausing of the polymerase allows for AID deposition on the DNA.

Whereas regions of weak secondary structure were not demonstrated to be highly mutable, the strongest predictor of hypermutability in transgenes was, in fact, primary sequence: the hottest nucleotide triplets were mutated most frequently, and, if true hotspots were lacking, the least cold of the coldspots were preferred [29•]. Incidentally, this very loose triplet sequence preference is also exhibited by the AID protein *in vitro*: AID can deaminate hotspots or coldspots in short oligos equally well, but when a long stretch of ssDNA is presented to the protein it seems to show mild preference for hotspots (although neither experiment was done in the context of transcription, and so the relevance of these findings is not clear). Overall, it is certain that the hypermutation machinery has local sequence preferences, and that these can be influenced by neighboring sequences [31]. The conclusion from *in vivo* studies is that transcription is essential, and experimental evidence from *in vitro* studies suggests that it may serve as an initial targeting mechanism for AID-mediated DNA deamination. But what, exactly, transcription provides to AID in terms of target sequence remains to be determined.

Higher order regulation of the somatic hypermutation reaction

If AID is indeed the initiator of a highly mutagenic reaction, the need for restricting its action to the appropriate areas of the genome becomes even more acute. How is that accomplished? Thus far we envision multiple controls for AID: it needs to enter the nucleus, and when it is there it can deaminate transcribed genes. But not all transcribed genes are mutated. Previous experiments showed that Ig enhancer elements are necessary for hypermutation. It is unclear whether the mutational effect of enhancers is position dependent (unlike conventional transcriptional enhancers; [21,32]) or position independent [33,34]. It appears likely that there is a maximum distance between enhancers and promoters beyond which they cannot effectively interact (which might account for the different experimental results). However, the well-defined Igκ intronic and 3′ enhancers are individually necessary [35], although together not fully sufficient [33], to target hypermutation to transgenic substrates, and other as yet unidentified sequences in the vicinity of the enhancers may play a role [33]. The 3′ lambda enhancer is sufficient to support hypermutation of a λ1 transgene [36], but the Ig heavy chain intronic enhancer does not drive efficient hypermutation of Ig heavy chain transgenes, even when these are heavily transcribed [37].

Traditionally, these experiments have been taken as evidence that sequences within the Ig enhancer serve as mutational enhancers that load the mutation initiation factor onto the transcription complex at the promoter. Yet, sequences important for mutation but not required for transcription were not known until recently, when Storb and colleagues [38] described experiments where the serendipitous creation of two E-box elements within the mutating V gene increased mutation several fold, whereas transcription was, if anything, reduced. It is not clear, however, if introduction of an E-box element in the mutated region itself is analogous to the effect of such a sequence in the enhancer. It remains to be seen whether addition of an E-box to a transgene that lacks Ig enhancer sequences will recruit hypermutation.

Specific chromatin structure at sites of somatic hypermutation

An alternative interpretation of the enhancer experiments is that the Ig enhancers create a specific chromatin configuration that is attractive to AID. Overexpression of AID leads to hypermutation in all organisms tested, but, despite high protein levels, the rates of mutation are fairly low. Even more significantly, very few clones in these experimental systems actually hypermutate, although AID is uniformly present and the target regions are transcribed at high rates [5•]. Thus, overexpression of AID may indeed lead to mutation, but only in a stochastic fashion, perhaps only in a small number of clones that happen to have acquired (or shed) certain epigenetic modifications that shield the rest of the otherwise indistinguishable clones. CSR provides a good paradigm for the differential protection of even adjacent DNA regions from the hypermutation machinery. It has been shown that high rates of transcription and expression of AID protein are required for both CSR and SHM. That, however, presents a paradox, for in the presence of AID, the transcribed switch region mutates and recombines, but at the same time the equally well transcribed variable region does not mutate, even though these sequences are only a few kilobases apart and are, in fact, components of the same primary transcript. So, what protects V regions from further mutation during CSR? Work from Liu, Banchereau and colleagues [39] has suggests that SHM temporally precedes CSR. Although this has not been conclusively proven, it makes theoretical sense that specificity is fixed by mutation before the switch to a different constant region, and it is possible that post-mutational, epigenetic modifications of the V regions

shield that part of the gene from further mutation. Such modifications could include histone acetylation, phosphorylation or methylation [40]. To date, no one has looked for such modifications in V and S regions, but as they are actively transcribed, the prediction would be that both are equally hyper-acetylated.

Conclusions

In the past two years strong circumstantial evidence has emerged for the role of AID as a DNA mutator. Yet, overall, it is not clear what targets AID predominantly to the transcribed Ig locus. Indiscriminate deamination has the potential to damage the genome: AID overexpression leads to T-cell tumors in mice [41•], and high expression of AID is a distinctive feature of certain aggressive B-cell lymphomas in humans [42]. We expect that, in the next few years, several questions about the regulation of hypermutation will be answered. Some of the most important research endeavors will be to identify how AID is regulated (what signal cascades are involved), what targets it to the correct loci (particular sequences or certain chromatin modifications) and, finally, what turns the entire reaction off?

Update

Recently, chromatin immunoprecipitation experiments have confirmed that AID associated with CSR target sequences in a transcription-coupled manner [43].

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