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AID targeting is dependent on RNA polymerase II pausing

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Abstract

Activation induced deaminase (AID) is globally targeted to immunoglobulin loci, preferentially focused to switch (S) regions and variable (V) regions, and prone to attack hotspot motifs. Nevertheless, AID deamination is not exclusive to Ig loci and the rules regulating AID targeting remain unclear. Transcription is critically required for class switch recombination and somatic hypermutation. Here, I consider the unique features associated with S region transcription leading to RNA polymerase II pausing, that in turn promote the introduction of activating chromatin remodeling, histone modifications and recruitment of AID to targeted S regions. These findings allow for a better understanding of the interplay between transcription, AID targeting and mistargeting to Ig and non-Ig loci.

Keywords

AID targeting; Germline transcription; Epigenetic modifications; RNA polymerase II pausing

1. Introduction

Immunoglobulin (Ig) antigen binding specificity is determined by assembly of a mature V region via V(D)J recombination during early B cell development. Antibody binding to antigen undergoes affinity maturation by means of somatic hypermutation (SHM), a mutational process, that is focused to rearranged V(D)J region genes in germinal center B cells. IgH effector function, determined by constant (C_H) genes, is also diversified in mature B cells through class switch recombination (CSR), while maintaining the original antigen binding specificity arising from V(D)J recombination. The mouse immunoglobulin heavy chain (*Igh*) locus contains a series of eight constant (C_H) genes (μ , δ , $\gamma 3$, $\gamma 1$, $\gamma 2b$, $\gamma 2ba$, ϵ , and α) that are located downstream of the V, D and J_H segments (Fig. 1A). Each C_H gene, with the exception of $C\delta$, is paired with a repetitive DNA switch (S) region and is a target for CSR. CSR involves the introduction and joining of double strand breaks (DSBs) in $S\mu$ and a downstream S region with the concomitant deletion of the intervening genomic material and generation of $S\mu$ –SX hybrid junctions. Activation induced deaminase (AID) deaminates dC residues in transcribed V(D)J exons or S regions and is critical for both SHM and CSR, respectively [1,2]. The means by which DNA lesions or DSBs initiated by AID deamination are repaired and culminate in SHM at V(D)J exons or CSR targeted to S regions have been extensively reviewed elsewhere [3–11].

The requirement for transcription is a hallmark of SHM and CSR [12,13]. V_H region transcription is directed from a promoter 5' of the rearranged V_HDJ_H exon and terminates downstream of the $C\mu$ exons. In V_HDJ_H exons, AID induced mutations are most prevalent

within 2 kb of the transcription start site (TSS) and are rectified by error prone repair [3,8,11,13]. These and other studies suggest that the transcription apparatus or transcription per se is a requirement for the process of SHM [14–16]. For CSR, a transcriptional promoter coupled with an I (intervening) exon is situated immediately upstream of a S region paired with each C_H gene [17]. Transcription initiates at the 5' end of the I exon, proceeds through the S region and terminates downstream of the corresponding C_H gene (Fig. 1A). The transcribed mRNA products are termed sterile or germline transcripts (GLTs) since they lack an open reading frame and are not translated [6,17]. Combinations of specific cytokines and B cell activators stimulate transcription from particular GLT promoters and target the paired S region for CSR [17]. Gene targeting studies have highlighted the critical requirement for genetic element directing GLT expression as a prerequisite for CSR [18].

At the primary DNA sequence level, AID deamination occurs preferentially at the WRC motifs (W = A/T, R = A/G) (reviewed in [19]). WRC motifs are scattered throughout the genome but AID deamination is preferentially focused to Ig genes. Nevertheless, AID mistargeting to non-Ig genes is nonrandom and occurs at measurable frequencies in a set of highly transcribed genes [20,21]. In vivo, AID induces DSBs at hundreds of non-Ig sites many of which are syntenic with sites of translocations, deletions, and amplifications found in human B cell lymphomas and are not necessarily restricted to transcribed regions [22]. AID targeting becomes promiscuous upon ectopic overexpression [23–26] or as a consequence of environmental events that elevate AID expression [27]. Aberrant AID targeting may be responsible for SHM of non-Ig genes in B cell tumors [28] and *Igh:myc* translocations in AID expressing mature B cells [29,30]. Mutations in TP53 gene accumulate following infection of human gastric epithelial cells with *Helicobacter pylori* which causes induction of AID expression [31]. The preferential focus of AID to Ig loci and the nonrandom aspect of AID attack on non-Ig genes imply a determinant set of rules governing AID targeting. Here, I explore new integrating principles regarding the mechanism of transcription, its relationship to epigenetic chromatin modifications, chromatin accessibility, and its influence on three dimensional *Igh* chromatin organization, which together provide new insights regarding AID targeting in CSR and SHM and the potential for genomewide mistargeting.

2. Formation of the S/S synaptosome relies on transcriptional elements

S regions targeted for recombination are dispersed over 220 kb and this distance will impede formation of S/S synaptosome required for CSR. Chromosome conformation capture (3C) techniques permit the quantitative measurement of long range interactions along chromatin fibers [32,33]. Studies of the *Igh* locus using 3C methods have made two important observations pertinent to CSR. First, in the *Igh* locus the E μ enhancer directly interacts with the downstream 3'E α LCR to form a unique chromosomal loop in vivo [34–36] (Fig. 1B). Second, inducible long range intra-chromosomal interactions between the GLT promoter region and the downstream 3'E α [34,36] are in accord with the requirement for 3'E α element to enable inducible GLT expression [37,38]. The effect of simultaneous E μ : 3'E α :GLT promoter interactions is that S μ , proximal to E μ is also in close contact with a downstream S region located adjacent to its GLT promoter (Fig. 1C and D). This three dimensional spatial organization of S regions targeted for CSR assures formation of the S/S synaptosome required for successful recombination and highlights the highly integrated requirements of CSR for transcription.

3. Transcription and the distribution of AID induced mutation

The distribution of mutations across V(D)J exons has a sharp 5' boundary located about 120 bp downstream of the TSSs but a less defined 3' boundary approximately 1 kb downstream

of the promoter [39,40]. Promoter proximal transcription has been strongly linked to induction of AID dependent mutations because alteration of the promoter position leads to a corresponding displacement of transcription initiation and perturbation of mutation distribution [12,41]. Consequently, Peters and Storb hypothesized that mutations are introduced by a mutator, now known to be AID, that associates with the transcription initiation apparatus, tracks with the transcription elongation complex and dissociates stochastically [12]. This model was later applied to CSR, because similar to the mutation pattern found for V exons, S region mutations begin 150 bp downstream of the I exon transcription start site [42]. However, the 3' boundary for CSR mutations is located downstream of S regions, a distance of up to 10 kb from the TSS, a distribution distinct from that found for SHM [42]. Recent studies support the Peters and Storb hypothesis and indicate that ectopically expressed AID binds with RNAP II in co-immunoprecipitation assays [43] and interacts with the transcription apparatus in vitro [44] implying that AID targeting is closely aligned with transcription.

4. Regional targeting of AID to S regions and exclusion from C_H regions

AID targeting is strikingly asymmetric within the I-S-C_H region. Whereas S regions are substrates for AID attack as evidenced by acquisition of mutations and DSBs, C_H regions within the same transcription unit are protected [42,45,46]. Studies concerning transcription through chromatin provide a mechanistic perspective on how specific DNA targets become differentially accessible. Eukaryotic DNA is wrapped around histone octamers to form nucleosomes that are organized into higher order chromatin fibers which precludes access of trans acting factors to DNA [47–49]. Eukaryotic transcription begins at the promoter with the assembly of the preinitiation complex (PIC) composed of hypophosphorylated RNA polymerase II (RNAP II) and general transcription factors followed by nucleosome displacement, chromatin accessibility, and transcription initiation [49–52]. Early elongating RNAP II clears the promoter and transits to the promoter proximal pause site. The paused RNAP II complex becomes increasingly phosphorylated at Ser5 (p-ser5) on the C-terminal domain (CTD). RNAP II escapes from the pause site by entering productive elongation whereupon the CTD loses p-ser5 and becomes enriched for p-ser2. There is an important link between the phosphorylation status of RNAP II CTD, the recruitment of histone modifying enzymes and acquisition of chromatin hyperaccessibility in S regions [48,49,53,54].

Genome wide analyses indicate that in transcriptionally active genes, promoter proximal sites are enriched for tri-methyl histone H3 lysine 4 (H3K4me3), hyper-acetylated (Ac) H3K9, and with RNAP II p-ser5 whereas, H3K36me3 and elongating RNAP II p-ser2 are elevated in the downstream coding regions [55–58]. H3K4me3 is directly bound by a component of the NuA3 histone acetyltransferase (HAT) complex that coordinates transcription activation with histone Ac (reviewed in [59]). DNA accessibility increases in response to histone Ac which changes the net charge of nucleosomes and alters chromatin fiber folding properties [60]. Histone Ac may also create binding surfaces for factor-histone contacts that then facilitate recruitment of transcription regulators [48]. In yeast, H3K36me3, is recognized by the histone deacetylase complex (HDAC), Rpd3S, which acts to diminish histone Ac and thereby repress transcription initiation in downstream coding regions [61,62]. Thus, H3K4me3 and H3K36me3 modifications provide for recruitment of HATs and HDACs, respectively, which in turn modulate chromatin accessibility to assure spatially appropriate transcription initiation within the transcription unit. Collectively, these studies have significant implications for understanding the AID targeting to hyperaccessible S regions and the exclusion of the C_H regions from AID attack.

Within the Igh locus, transcribed I-S regions are a focus for increased H3K4me3 and histone Ac while CH regions accumulate the repressive countermarks, H3K36me3 and H4K20me1 [43,63–65]. In chromatin, I-S regions undergoing active transcription are nuclease hypersensitive whereas C_H regions are relatively inaccessible [63,64]. Antisense RNA transcripts are detected in S regions but not in C_H regions reflecting the reciprocal zones of accessible or repressed chromatin [66]. Modulation of H3Ac at S regions using trichostatin A, an inhibitor of HDACs, leads to greater chromatin accessibility and increased CSR indicating a direct link between this histone mark and switching frequency [63]. B cells with PTIP (PAX interaction with transcription activation domain protein-) deficiency display reduced H3K4me3 and histone Ac marks and impaired transcription initiation at several downstream S regions [67]. PTIP is a constituent of several histone methyl transferases (HMTases) including MLL3 (mixed lineage leukemia 3)–MLL4 complex that associate with RNAPII p-Ser5 and introduce H3K4me3 modifications [67,68]. PTIP also indirectly interacts with p300/CBP HAT suggesting that PTIP regulates both histone Ac and Me [69]. These findings support the conclusion that histone Ac status and chromatin hyperaccessibility are patterned across the I-S regions by means of H3K4me3 modifications linked to transcription. Long range interactions between GLT promoters and the 3'Ea enhancer are impaired in PTIP deficient B cells which reflects the failure of GLT transcription initiation [70].

5. S region sequence mediates RNAP II occupancy

S regions extend 1–10 kb downstream of the I exons [71], and when transcribed are decorated with H3K4me3, H3Ac, H4Ac, activating histone modifications, and reside in hyperaccessible chromatin throughout their lengths [63,64]. In contrast, genome wide ChIP-seq studies show that these histone marks are largely restricted to promoter proximal locations and rarely extend more than 1 kb downstream of the TSS [55,56]. The observation that RNAP II p-ser5 collaborates with an HMTase to introduce H3K4me3 at the 5' end of active genes [55,56,72] suggested that the unusual distribution of H3K4me3 in S regions might be explained by stalled RNAP II p-ser5. ChIP studies demonstrated a significant enrichment of RNAP II p-ser5 throughout the S_μ and S_{γ3} regions implying that RNAP II has stalled at multiple sites in S DNA [63]. In S_μ deleted B cells, RNAP II p-ser5 occupancy and H3K4me3 marks remained promoter proximal, demonstrating that S region sequence mediates RNAP II enrichment [63,73]. DNA breaks inhibit transcription by RNAP I [74] but are not the cause of high occupancy RNAP II since retention in S regions occurs equivalently in AID proficient and deficient B cells [63].

Transcribed S regions are unique in that they contain long stretches of R loops that enhance CSR efficiency in vivo [75,76]. R-loops are composed of RNA:DNA hybrids hybridized to the template strand, impede transcription elongation [77,78] and could be responsible for RNAP II stalling detected in S regions. Enrichment of RNAP II in S regions could occur by another non-mutually exclusive mechanism. Broadly distributed H3K4me3 is detected across multiple Hox-coding and intergenic regions, and is correlated with the RNAP II transcription of noncoding RNAs [55,58]. Accordingly, anti-sense RNAs have been detected immediately 5' of transcriptionally active S regions [66]. It is possible that antisense RNA expression initiate from multiple alternative promoters within the S regions, attract RNAP II which becomes stalled and hyperphosphorylated at p-ser5, then recruits PTIP to introduce H3K4me3. Thus, there are two plausible mechanisms to account for the unique distribution of RNAP II p-ser5 in S DNA. The presence of sense and antisense transcription could create head to head collisions between RNA pol II molecules that in turn might amplify RNA pol II stalling.

6. The R-loop dilemma

AID activity is specific for ssDNA but lacks activity on dsDNA [79–82]. In vivo, AID deaminates dC residues nearly equivalently on both strands of DNA raising the question of how access is gained to the transcribed strand (reviewed in [3,6,11,19]). SHM and CSR require transcription through dsDNA to generate ssDNA template which could be targeted by AID. During GLT expression R-loops are formed when the transcribed template strand hybridizes with the nascent S region transcript and the nontemplate strand is looped out as a long stretch of ssDNA [75,76,83]. Biochemical studies indicate that transcription generated R-loops within dsDNA allow DNA deamination primarily on the ssDNA nontemplate strand [82]. AID, phosphorylated on serine 38 by protein kinase A, can access transcribed dsDNA substrates that lack R-loops, as for V(D)J exons, but primarily on the nontemplate strand [84] (see also Vuong and Chaudhuri, this issue). Ectopic expression of AID in bacteria or yeast introduces mutations, mainly on the nontemplate strand [80,85]. RNAP II generated supercoiling occurs immediately upstream and downstream of the transcription complex [86]. Certain transcribed plasmids acquire AID induced mutations on both strands due to supercoils [87]. However, this does not explain how AID accesses the template strand when it is blocked by the nascent RNA transcript that will form the R-loop [19].

Evidence indicates that the RNA exosome, a cellular RNA-processing degradation complex, associates with AID, accumulates on S regions in an AID dependent manner and is required for CSR [88]. The RNA exosome complex contains 3′-5′ exoribonucleases that are implicated in the processing of structural RNA and in the degradation of improperly processed pre-mRNA [89]. The RNA exosome is tethered to the RNAP II via the transcription elongation factors Spt5 and Spt6 [89]. Biochemical analyses demonstrate that the RNA exosome core complex facilitates AID- and transcription dependent deamination to both the template and nontemplate strands in vitro [88]. Phosphorylation of AID on Ser38 is a precondition for association with replication protein A (RPA) a complex which binds ssDNA as a trimolecular ring [90,91]. RPA associates with the S region prior to AID binding. It has been suggested that the ssDNA targets generated on the template strand by the RNA exosome might be stabilized by RPA which in turn mediates the accumulation of AID p-Ser38 by direct interaction [88] (see also Vuong and Chaudhuri, this issue).

7. AID targeted to paused RNAP II in S regions

Is RNAP II pausing simply a byproduct of transitioning through difficult R-loop territory? Or, does RNAP II pausing in S DNA confer some advantage to CSR? Transcription initiation begins with clearance of RNAP II from promoter bound factors and is then paused 25–50 nucleotides from the TSS by 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) sensitivity inducing factor (DSIF) composed of Spt4 and Spt5, and negative elongation factor (NEF) [50]. Current models suggest that positive transcription elongation factor b (P-TEFb) kinase drives the transition from the paused to the actively elongating state by phosphorylating RNAP II and Spt5 leading to the release of NELF [50,53]. A shRNA screen recently identified Spt5 as a critical regulator of CSR [92]. Co-immunoprecipitation studies demonstrated that Spt5 interacts with AID and facilitates association with RNAP II [92]. ChIP-seq experiments show a strong correlation between positions of Spt5 and RNAP II stalling [92,93] and between sites of Spt5 and AID binding genomewide [92]. Technical concerns regarding the quality of the AID ChIP-seq and its interpretation, however, make this aspect of the study somewhat controversial. Collectively, the data suggest that AID accumulates at sites of RNAP II pausing by means of its association with Spt5. The high density of paused RNAP II and Spt5 in transcribed S regions appears to confer preferential AID targeting to these sites.

8. Links between Spt5, co-transcriptional factors and CSR

Earlier studies showed that pre-mRNA splicing may be integral to CSR since deletion of the GLT γ 1 I exon splice donor abolished $\mu \rightarrow \gamma$ 1 switching [94,95], however the mechanism linking RNA processing to CSR was obscure. Transcription proceeds through a series of transitions moving from initiation, early elongation, promoter proximal pausing, active elongation and termination with pre-mRNA processing and splicing occurring as a series of functionally coupled co-transcriptional events [50,96,97]. Spt5 interacts with a number of co-transcriptional factors, and may thereby serve as a functional adaptor to the transcription machinery. Spt5 links RNAP II to splicing factors [98], capping enzyme [99,100] and the RNA exosome [89]. Accordingly, several RNA processing and splicing factors have been functionally linked to CSR and could be recruited to S DNA via Spt5. Polypyrimidine tract binding protein 2 (PTBP2), identified based on its biochemical association with AID, is a critical regulator of CSR [101]. PTBP2 binds S region RNA transcripts and DNA, and targets AID to S regions [101]. PTBP2 is highly homologous with the well characterized PTBP1, a regulator of splicing that binds RNA polypyrimidine tracts, blocks spliceosome assembly and facilitates exon exclusion during alternative splicing [102,103]. CTNNBL1, a spliceosome associated factor, interacts with AID in vivo and in vitro and may have a partially redundant role in nuclear trafficking or retention of AID [104,105]. Although targeted deletion of CTNNBL1 has little effect on CSR [106] this may be due to other proteins which facilitate AID nuclear transport [107]. Together, the data suggest that Spt5 is a critical adaptor which anchors co-transcriptional factors to RNAP II and recruits AID to the transcriptional complex. It is intriguing to speculate that the I exon associated splice donor acts as an anchor for the co-transcriptional machinery and is required for appropriate association of Spt5 with RNAP II. These findings suggest a multilayered relationship between Spt5 and paused RNAP II and/or binding of co-transcriptional factors to enable recruitment of AID to S regions. However, this model must be considered incomplete since other factors modulate AID binding to S DNA including, KRAB domain-associated protein 1 (KAP1) [108] and the 14-3-3 scaffolding protein [109]. It is currently unclear whether KAP1 and 14-3-3 are related to RNAP II pausing or other co-transcriptional activities.

9. Cis-regulatory elements as recruiters for AID

Transcriptional elements are candidates for providing specificity for AID attack. Transcription of Ig H and L chain genes is driven by well characterized promoters and enhancers [110], however, a specific role for each in SHM has been difficult to define. AID attack of Ig loci has been shown to occur in close proximity to either the V [3,19] and S region promoters [42]. Earlier studies showed that heterologous promoters functioned well for both SHM and CSR suggesting that these transcription elements are fungible [3,95]. Evidence also indicates that mutation frequency correlates with transcription rates in manipulated transgenes [3,19]. However, substitution of the endogenous Ig promoter with the highly transcribed elongation factor 1- α promoter leads to a reduction in SHM in the chicken DT40 B cell line indicating that not all promoters are equally capable of supporting SHM [111]. An enhancer has been identified within the DT40 IgL locus that confers mutability on heterologous genes and is distinct from the known transcriptional enhancer elements [112]. Targeting of AID activity to Ig loci may occur by species specific mechanisms since the murine IgLk transcriptional enhancer located in the context of the DT40 IgL locus supports transcription but not SHM [113].

Recently, the E-box motif, CAGCTC, a binding site for the transcription factor E2A, was shown to facilitate SHM in a GFP mutable transgenic substrate driven by a CMV promoter and the mouse Lk intronic enhancer [114]. These findings suggest that E-box motifs and E2A binding collaborate with the Ig enhancer to specify AID attack, although the method by

which this occurs remains unclear. Intriguingly, E-box motifs are found at high density in S regions targeted for CSR and engage with E2A factors in vitro [115]. More work is required to clarify the involvement of E2A in AID targeting.

10. Concluding remarks

Models seeking to understand AID targeting must incorporate the observation that AID both focuses preferentially to Ig loci but also targets other non-Ig genes. Chromatin remodeling, histone modifications and accessibility in S regions are linked to transcriptionally active but paused RNAP II. AID may be preferentially recruited to the S region by means of several variables which in combination provide a unique context. RNAP II pausing in S DNA may be severe due to R-loop formation, which is an intrinsic feature of transcribed S regions. The expanse over which RNAP II pausing occurs is long, 1–10 kb, and may provide a platform for substantial Spt5 recruitment. AID may also be recruited by PTBP2, 14-3-3 and KAP1, which bind to transcribed S regions. The compounding effect of these individual events may provide the specificity required for AID targeting to S regions. Thus, non-Ig genes with several of the features characteristic of S regions may attract AID at lower but still detectable levels. The mechanism by which AID attacks V genes during SHM is unclear because V genes lack R-loops. It will be of great interest to determine whether RNAP II pausing occurs in V genes as a necessary feature of SHM.

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Fig. 1.

AID creates DSBs in a looped *IgH* locus. The diagram (not to scale) shows VDJ exons (blue box), the intronic μ enhancer, E_μ (yellow circle), S_μ , $S_\gamma 3$, $S_\gamma 1$ and $S_\gamma 2b$ regions (orange, red, green and blue ovals, respectively), I exons (black rectangles), C_H genes (gray boxes) and the 3'E α regulatory regions (gray filled circle). (A) Following B cell activation with LPS and IL4 both the μ and $\gamma 1$ GLTs are expressed. (B) In resting B cells the *IgH* locus is in a looped configuration tethered by long range interactions between E_μ and 3'E α . (C) Upon treatment with LPS and IL4 the $\gamma 1$ GLT promoter interacts with 3'E α and brings $S_\gamma 1$ into proximity with S_μ . (D) B cell activation also activates AID that in turn generates DSBs in S_μ and $S_\gamma 1$. The DSBs are resolved by a reciprocal recombination event which generates an excision circle and a new hybrid $S_\mu/S_\gamma 1$ junction along the chromosome, thereby facilitating IgG1 expression.