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Epigenetic regulation of antigen receptor gene rearrangement

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Summary

Recent studies of the regulation of antigen receptor rearrangement have revealed several completely new levels of control. Not only do antigen receptor loci undergo changes in histone modifications as they become accessible for recombination, but the number of different histone modifications and the variation at different parts of each receptor locus reveal great complexity. RAG2 is now known to bind to one of these histone modifications, H3K4me3, and this targets the initial RAG binding events to the J genes. The large megabase receptor loci undergo 3-D changes in their structure during rearrangement, and receptor loci move throughout the nucleus, transiently binding to heterochromatin, and transiently pairing with each other. RAG-mediated DNA breaks promote some of these movements, and also result in widespread changes in the transcriptional profile promoting differentiation.

Introduction

The V(D)J rearrangement process provides us with a highly diverse collection of antibodies and T cells to combat a wide variety of pathogens. This process rearranges V, D and J genes located in 6 distinct complex receptor loci, and it is under precise T vs. B lineage specificity. In addition, there is tight developmental stage specificity of the recombination process, with individual loci recombining in an ordered manner. Furthermore, within a locus, D to J recombination always occurs before V to DJ gene rearrangement. Alt and colleagues proposed the “accessibility hypothesis” to explain this complex regulation, and this model has been confirmed by extensive experimental support over the years, most strongly by the experiments demonstrating that *in vitro* cleavage at RSS within intact nuclei recapitulate the lineage- and stage-specificity observed *in vivo* [1,2]. In the intervening 25 years since this hypothesis was first proposed, we have learned a lot more about the epigenetic, transcriptional and 3-dimensional regulation of the complex process of V(D)J recombination, and I will review the current status in this article.

Epigenetic changes at histone tails at antigen receptor loci

The core unit of chromatin is the nucleosome, which consists of an octamer of histone proteins (Fig. 1). The tails of the histone proteins are subject to a variety of post-translational modifications. Genes in receptor loci that were actively undergoing recombination have been shown to be associated with acetylated histones and other histone posttranslational

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modifications associated with active genes, whereas the histones did not have these marks in loci that were not yet recombining [3–7]. However, it is too simplistic to think of chromatin as either open and accessible or closed and inaccessible. The extent of specific histone modifications can be very different on V, D or J genes even when all of the genes are poised for recombination. For example, H3K4me3 is restricted to J genes and a few very closely located D genes, while dimethylation of H3K4 is more widely spread among V, D and J genes of poised loci [4,8–10]. Although V and D genes can be associated with acetylated histones, the level of acetylation is far higher on J genes than on any V genes, even during the stage of V_H to DJ_H rearrangement [4,9].

RAG1 and RAG2 as “readers” and “writers” of histone modifications

More recently, it has become appreciated that the predominant consequence of these various histone modifications was to act as docking sites for recruitment of complexes containing chromatin modifying proteins and/or transcription factors to that region (recruiting “readers” of epigenetic marks)[11]. One very exciting example of this with high relevance for V(D)J recombination was the finding that the PHD domain in the non-core portion of RAG2 specifically bound to H3 when it was trimethylated at lysine 4 (Fig. 1). Furthermore, mutations in the PHD domain that abolish binding H3K4me3 resulted in greatly impaired VDJ recombination activity [8,12]. Since antigen receptor loci that are rearranging have high levels of H3K4me3 on the J genes, this predicts that RAG2 would be directly recruited to J genes [8,9]. Preferential binding of RAG2 to J genes suggested a mechanism by which ordered recombination of D to J before V to DJ could be achieved [9,10]. In elegant demonstration of the validity of this hypothesis, Ji et al. have shown by ChIP that RAG2 binds to J genes at all of the receptor loci in a lineage specific manner, as well as binding to H3K4me3 throughout the genome of these cells [10]. In contrast, RAG1 binds only to RSS at J genes within the active loci. Thus, the RAG1 and RAG2 binding pattern results in ordered DJ before V to DJ rearrangement in a lineage-specific manner, and these studies directly link epigenetic changes in the receptor loci to recruitment of the recombinase. Although this data help explain the mechanism by which lineage restriction and the beginning of ordered rearrangement is controlled, it was observed that RAG1 and RAG2 continues to bind to the J_H and J_β genes in pre-B and double positive (DP) thymocytes, respectively, suggesting that the control of allelic exclusion, and cessation of rearrangement of IgH and TCR β genes at the second allele at the subsequent stage of development is not controlled by accessibility of the J genes. Rather, it is likely to be controlled at the level of accessibility of the V gene locus as described in more detail below. In addition to targeting RAG2 to the J genes, H3K4me3 serves an additional function in that binding of H3K4me3 to RAG2 increases the enzymatic activity of the RAG complex [13].

RAG2 is not alone in its epigenetic functions. RAG1 has a RING finger in its non-core region, and this domain functions as an E3 ubiquitin ligase (making it an epigenetic “writer”) [14]. It can ubiquitinate histone H3 and non-histone proteins *in vitro*, and it undergoes auto-ubiquitination *in vivo* [15]. Very little is known about any physiological substrates of this E3 ligase activity. However, the fact that mutations in the RING finger that reduce catalytic activity *in vitro* result in decreased recombination of a transfected extrachromosomal recombination substrate, and furthermore, that some of these ligase mutations have been found in Omenn’s Syndrome immunodeficiency patients, strongly suggests that the E3 ubiquitin ligase activity is likely to be of functional significance for V(D)J recombination [16,17].

Germline transcription: first sense and now antisense too

It has been known for over 25 years that unrearranged V gene segments and J-C regions are transcribed in a lineage- and stage-specific manner [18,19]. However, it remained unclear for a long time whether germline transcription was required to establish accessibility, or whether it was merely a byproduct of the fact that the region had been made accessible. Strong evidence that germline transcription was required for rearrangement came from a study in which a transcriptional terminator element was targeted into the $J\alpha$ locus [20]. This abrogated transcription of several $J\alpha$ genes downstream of the terminator, and it also abolished recombination to those $J\alpha$ genes. Importantly, this same region also showed greatly reduced levels of histone acetylation and H3K4me3. Since histone methyltransferases that methylate H3K4 often travel with the RNA polymerase II complex, and since RAG2 is recruited to H3K4me3, these data suggest that germline transcription is important for inducing accessibility by adding the H3K4me3 mark onto histones surrounding J genes. It should be noted that the germline transcription through J-C regions is the most easily detectable germline transcription at all receptor loci, which may account for the fact that J genes are enriched in H3K4me3 and are the first genes to undergo rearrangement.

A newer role for germline transcription in influencing accessibility emerged with the discovery by Corcoran and colleagues that there is extensive antisense transcription throughout the large V_H region of the IgH locus [21]. This antisense transcription is even more strictly regulated than sense germline transcription, in that it first appears after DJ_H rearrangement, and ceases after V_H to DJ_H rearrangement. Based on this timing, it was proposed that the antisense transcription serves to open the V_H locus for V_H gene rearrangement. Subsequently, the Corcoran lab and the lab of Ranjan Sen both demonstrated that antisense transcription also occurs in the D_H locus, and the timing of this antisense transcription suggests that it promotes accessibility for D_H to J_H rearrangement [22,23]. Chakraborty et al. further suggest that D_H antisense transcripts may pair with the low level of sense transcripts from the DSP genes resulting in Dicer-dependent repression of rearrangement of the DSP genes [23]. In support of this hypothesis, Dicer ablation in the germline results in an antibody repertoire with higher usage of DSP genes [24]. These long antisense D_H and V_H transcripts are likely to play a key role in regulating accessibility for recombination.

Chromatin remodeling and enhancers

In addition to probably adding the H3K4me3 mark, the act of transcription may disrupt nucleosomal structure, at least transiently. It has been demonstrated that *in vitro* assembly of RSS-containing DNA into a nucleosomal structure will reduce the ability of that DNA to undergo RAG-mediated cleavage [25,26]. Thus, it is likely that ATP-dependent chromatin remodeling may be necessary to make regions of DNA accessible to RAG binding and recombination [27,28]. In this regard, it has been shown that SWI/SNF complexes are recruited to the J_H genes in an $E\mu$ -dependent manner, and knockdown of the ATPase subunit of SWI/SNF results in reduction of antisense transcription, which begins near $E\mu$ [28]. This suggests that $E\mu$ may initiate accessibility by recruiting the SWI/SNF chromatin remodeling complex, and that this recruitment is necessary for antisense transcription to make the D_H - J_H region accessible [28]. Germline deletion of $E\mu$ greatly reduces histone acetylation on D_H and J_H genes, reduces H3K4me3 levels on J_H genes 3-fold, and reduces germline transcription, both sense and antisense, within the D_H - J_H region [29]. Coincident with loss of all of these indications of accessibility, deletion of $E\mu$ greatly reduces IgH rearrangement [30,31]. In contrast to the D_H - J_H region, sense and antisense transcription are only reduced in the most proximal V_H genes upon $E\mu$ deletion. Thus, $E\mu$ controls accessibility for ~400

kb upstream [23]. Likewise, E α acts in a very similar pattern in controlling histone modifications and germline transcription of V α genes for a distance of up to 500 kb from E α , but not throughout the distal portion of the TCR α locus [7].

Nuclear repositioning and 3-D changes in the structure of receptor loci

The pioneering study of Kosak et al. showed that Ig receptor loci are located at the nuclear periphery in non-B cells, and they become centrally located in pro-B cells [32]. The nuclear periphery is thought to be a repressive environment, and this group has more recently directly demonstrated that targeting genes to the nuclear lamina does indeed result in repression [33]. In addition, in their earlier study, they made the surprising discovery that the IgH locus becomes compacted at the time of rearrangement [32]. Subsequent 3D-FISH studies by several labs have confirmed this finding, and extended it to the Ig κ and TCR β loci [34,35]. The TCR α/δ locus is the most complex locus, with the TCR D δ , J δ , and C δ genes being located upstream of the J α genes and downstream of the intermixed V α and V δ genes. TCR δ genes rearrange at the double negative (DN) thymocyte stage, while TCR α rearrangement occurs later in the double positive (DP) stage. Recently, detailed 3D-FISH studies demonstrated that the most distal part of the TCR α/δ locus is contracted only in DN cells whereas the central portion of the locus and the J α region is equally compacted in DN and DP thymocytes [36]. Since TCR δ rearrangement utilizes V genes scattered throughout the large V α/δ locus, it is logical that contraction of the entire locus would be necessary for the rearrangement of distal V δ genes in DN thymocytes. On the other hand, distal V α do not rearrange until very late in the DP stage. Rather, the most 3' V α genes rearrange with the most 5' J α genes, the cells attempt to pass positive and negative selection, and if that fails, rearrangement proceeds outward in subsequent rearrangement events, using progressively more 5' V α genes and more 3' J α genes. Thus, this complex pattern of locus contraction fits precisely with the distinct rearrangement patterns of TCR δ vs. TCR α genes.

An elegant study examining the 3D structure of the IgH locus during locus contraction was performed by Jhunjhunwala et al. [37]. Measurement of spatial distances between many small 10 kb probes located throughout the IgH locus demonstrated that distal and proximal V_H genes were equidistant from the D genes specifically in the pro-B cell stage when the V_H genes are rearranging. Trilateration and triple-point measurements suggested that the locus is organized into clusters of loops forming rosette-like structures that become closely juxtaposed specifically in pro-B cells, but that are separate at other stages (Fig. 2). That this locus compaction is essential for rearrangement for most of the V_H genes is strongly supported by the fact that deficiency of Pax5, Ezh2, YY1, and most recently Ikaros, have all been associated with impairment in rearrangement of all V_H genes except the most proximal V_H gene family, and also with a lack of locus contraction [38–41]. Ezh2 methylates H3K27, and it has been shown that the proximal V_H genes are normally associated with H3K27me3 chromatin [42]. Furthermore, this repressive mark is absent in Pax5 deficient pro-B cells suggesting an interrelationship of these pathways [42].

It has been proposed by Degner et al. that CTCF, a ubiquitously expressed protein which has been demonstrated to form long-range chromatin loops at other large loci, may play a major role in the looping of the IgH locus during V(D)J rearrangement [43] (Fig. 2). In support of this hypothesis, they demonstrated by ChIP-chip that there are a large number of sites within the V_H portion of the IgH locus that are bound by CTCF, as well as two close sites just 5' of the most 5' functional D_H gene, and a large cluster at the far 3' end of the locus, within the 3' regulatory region [43]. It is of interest that the antisense transcription which goes through the entire D_H locus at the time of D-J rearrangement falls off just beyond the CTCF site upstream of DFL16 [44], and that the high level of histone acetylation around DFL16 also drops off beyond that CTCF site [23]. Both of these observations suggest a boundary

function for the DFL-flanking CTCF site. These two regions of CTCF binding at DFL and the 3'RR may create a domain, probably by looping, which will allow D-J rearrangement to occur in a physically distinct domain from the V_H regions, thus acting in concert with preferential RAG binding to J_H RSSs in promoting ordered rearrangement [10,43–45].

Allelic exclusion, chromosomal movement, and association with heterochromatin

The explanation for why VDJ rearrangement only occurs on one allele at a time is still not completely understood. It is generally held that for the IgH and TCR β loci which rearrange first, the constraints on continued rearrangement in pre-B cells or DP cells, respectively, occur at the level of lack of accessibility of the V locus. Yet even this is not well understood on a mechanistic level, as exemplified by the study of Jackson et al. in which insertion of the TCR α enhancer into the V β locus caused increased germline transcription and histone acetylation of nearby V β genes in DP thymocytes, yet those V β genes did not undergo rearrangement in the DP stage despite having all of these hallmarks of accessibility [46]. The study of Ji et al. demonstrated that the J_H region is still marked by H3K4me3 in the pre-B cell stage, and RAG2 and RAG1 are still bound to it [10]. The same is true for the J β region in DP T cells, yet rearrangements do not occur at these receptor loci at that time.

Since compaction of the large V gene regions are necessary for efficient V(D)J rearrangement of V genes throughout the locus, it follows that de-contraction of the locus may render distal V genes inaccessible for rearrangement. Indeed, it has been demonstrated that the IgH and TCR β loci de-contract in pre-B and DP thymocytes, respectively [34,35]. In pre-B cells, the IgH allele lacking the productive rearrangement is transiently associated with pericentric heterochromatin. Thus, it has been proposed by Skok and colleagues that locus de-contraction, and transient association with pericentric heterochromatin both aid to enforce allelic exclusion at the differentiative stage following the stage at which those loci normally undergo rearrangement [34].

Even during the stage at which rearrangement is occurring, large scale chromosomal movement may limit simultaneous V gene rearrangement on both alleles. For the kappa locus, it was demonstrated that one kappa allele became transiently associated with heterochromatin in pre-B cells. This allele was the allele that replicated later, and was not enriched in acetylated histones but was associated with HP-1 and Ikaros [47]. Presumably this transient monoallelic association in this repressive environment resulted in temporary inhibition of accessibility for recombination while the other allele was actively undergoing rearrangement. Likewise, in DN thymocytes, it has been reported that one TCR β allele [35], or even more than one TCR β alleles [48], were recruited to pericentric heterochromatin or the nuclear lamina [35]. This latter study demonstrated that insertion of the TCR α enhancer into the TCR V β locus resulted in less heterochromatin association of that allele, and enhanced rearrangement of the neighboring V β genes, in some cases resulting in a lack of allelic exclusion. Thus, it appears that transient relocation of the receptor loci to repressive environments can indeed result in decreased ability of V gene rearrangement [48].

Chromosomal pairing of IgH and Igk alleles

Crosstalk between the two Ig receptor loci in pre-B cells also aids in controlling rearrangement. Hewitt et al. demonstrated that in pre-B cells, the Igk allele associated with pericentric heterochromatin recruits the nonrearranged IgH allele to associate with it via a mechanism requiring 3'Ek [49]. This interaction stimulated the de-contraction of the IgH locus, thus preventing further rearrangement. More recently, Hewitt et al have demonstrated that the two IgH alleles are transiently paired in a process that was dependent upon RAG

binding, but not RAG-mediated DNA cleavage [50]. When DNA breaks at the RSS do occur on one allele, the authors suggest that this signals transient repositioning of the other allele to heterochromatin in an ATM-dependent manner. This dynamic feedback system thus would prevent V_H recombination from happening on both alleles simultaneously. However, these types of analyses are complex to analyze, as the authors discuss in the addendum to their paper. RAG-mediated double strand DNA breaks do more than just this feedback signaling. Bredemeyer et al. demonstrated that these DNA breaks induce a broad transcriptional program that includes many genes involved in lymphocyte development [51]. Thus, V(D)J recombination is not only necessary for lymphocyte development by creating the antigen receptor, but it also provides cues to induce transcription of genes necessary for lymphocyte differentiation.

Conclusions and future directions

The past few years have revealed several new layers in the elegant complexity and crosstalk that regulate V(D)J rearrangement. The fact that RAG2 is recruited by an epigenetic mark is now quite clear, and it remains to be determined if RAG1's E3 ubiquitin ligase activity also plays a role in regulating or recruiting RAG recombinase activity. The factors that regulate the changes in the 3-dimensional structure of these large receptor loci during recombination have yet to be fully characterized. The changes in subnuclear localization, transient recruitment to pericentric heterochromatin, and crosstalk between receptor loci on different chromosomes provide the opportunity for such architectural changes to regulate V(D)J rearrangement, in addition to the genetic, transcriptional and chromatin modifications that have been intensively studied over the past several years.

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Fig. 1. Epigenetic regulation of V(D)J recombination at the *Igh* locus. A. Structure of the *Igh* locus. B. The DNA is organized into nucleosomes with the DNA wound around the histone octamers (blue circles). Germline transcription occurs in the sense direction (straight red lines) and antisense direction (wavy red line) preceding D_H to J_H recombination. J_H genes have histone 3 trimethylated at lysine 4 (gold circles). C. RAG2 binds to the H3K4me3 associated with J_H genes, and D_H to J_H recombination ensues. D. After DJ rearrangement, antisense transcription occurs throughout the V_H regions. E. V_H to DJ_H rearrangement occurs.

Fig. 2.

Three dimensional changes at the *Igh* locus. Pre-pro-B cells have multiple rosette-like looping structures. The D_H and J_H genes and enhancers are distantly located from the V_H genes. In the pro-B cell stage, these compartments come together in a process known as locus compaction. There are many CTCF sites throughout the V_H locus (white squares) that may facilitate the large scale proteins. There are binding sites for a variety of other DNA binding proteins throughout the locus that may also play a role in locus compaction.