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Regulation of Antigen Receptor Gene Assembly by Genetic-Epigenetic Crosstalk

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Abstract

Many aspects of gene function are coordinated by changes in the epigenome, which include dynamic revisions of chromatin modifications, genome packaging, subnuclear localization, and chromosome conformation. All of these mechanisms are used by developing lymphocytes to regulate the assembly of functional antigen receptor genes by V(D)J recombination. This somatic rearrangement of the genome must be tightly regulated to ensure proper B and T cell development and to avoid chromosomal translocations that cause lymphoid tumors. V(D)J recombination is controlled by a complex interplay between cis-acting regulatory elements that use transcription factors as liaisons to communicate with epigenetic pathways. Genetic-epigenetic crosstalk is a key strategy employed by precursor lymphocytes to modulate chromatin configurations at *Ig* and *Tcr* loci and thereby permit or deny access to a single V(D)J recombinase complex. This article describes our current knowledge of how genetic elements orchestrate crosstalk with epigenetic mechanisms to regulate recombinase accessibility via localized, regional, or long-range changes in chromatin.

Keywords

Lymphocyte Development; V(D)J Recombination; Epigenetics; Chromatin; Transcription

1. Antigen Receptor Gene Assembly

Evolution has endowed mammals with a sophisticated immune system that protects us from a continually changing world of pathogens. A centerpiece of this flexible defense is the ability of B and T lymphocytes to generate enormous repertoires of antigen receptors (AgRs), which bind and tag pathogens for elimination by a diverse set of immune mechanisms. The AgR repertoire is clonally distributed among lymphocytes so that every B and T cell expresses a signature receptor, presenting a unique interaction surface for a restricted set of foreign epitopes.

AgR diversity is restricted to the variable regions of immunoglobulin (Ig) and T cell receptor (TCR) proteins. Diversity is generated early in lymphocyte development by an antigen-independent process, called V(D)J recombination. In brief, each precursor B and T cell generates its signature variable region exons by selecting and fusing one variable (V), one joining (J), and sometimes one diversity (D) gene segment from large arrays of these elements

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spread over considerable distances (up to 3 Mb) in each of the seven antigen receptor loci (Fig. 1A) [1–3]. Although the total number of rearranging gene elements is relatively small at any given *Ig* or *Tcr* locus (< 200), the overall number of different antigen receptors generated by V(D)J recombination exceeds 10^9 by most estimates.

This unusual process of somatic DNA recombination is mediated by the enzymatic components of V(D)J recombinase, called RAG1 and RAG2, which are expressed specifically in precursor B cells and thymocytes [4,5]. The RAG1-2 complex binds conserved recombination signal sequences (RSSs) that directly flank all AgR gene segments [6,7]. RAG binding to a compatible pair of RSSs activates its endonuclease function, which generates DNA double strand breaks precisely at each gene segment-RSS border. Subsequently, the broken chromosome is rescued and the selected gene segments are fused by the non-homologous end-joining (NHEJ) machinery [8–10]. However, RAG-mediated breaks in precursor lymphocytes are potentially dangerous with regard to cell viability and genome integrity. Indeed, defects in NHEJ block lymphocyte development and can produce genetic lesions that initiate the formation of many lymphoid tumors [11]. As such, a more comprehensive understanding of the mechanisms that target V(D)J recombinase is of significant scientific and clinical value.

A central mystery in our understanding of V(D)J recombination regulatory mechanisms emerged from classical studies showing that all steps of *Ig* and *Tcr* gene assembly employ a common recombinase [12–14], which uses relatively indistinguishable substrates (RSSs). Despite the use of a shared enzyme-substrate strategy, recombinase activity is clearly targeted to distinct AgR loci depending on cell lineage and developmental stage [1,15]. In precursor B cells, *Ig* but not *Tcr* loci are assembled by V(D)J recombinase, whereas the opposite holds true for thymocytes.

AgR gene assembly also has become an integral component of programs that drive lymphocyte development [16,17]. The earliest committed precursors, termed pro-B or pro-T (DN) cells, initially assemble their *Igh* and *Tcrb* genes, respectively, using a sequential process that first targets D-to-J then V-to-DJ recombination. In the case of T cell development, if a functional *Tcrb* gene is produced on either allele, the expressed protein is incorporated into a pre-TCR, which signals for termination of further *Tcrb* rearrangement [18,19]. This process, called allelic exclusion, ensures that only one functional version of each AgR component is expressed by each lymphocyte, enforcing receptor monospecificity.

Signals emanating from the pre-TCR also drive developmental progression from the pro- to pre-T (DP) cell stage, in which recombinase activity is redirected to the *Tcra* locus [20]. Assembly of a functional *Tcra* allele permits expression of the mature TCR α / β surface receptor, which guides the thymocyte through positive and negative selection by MHC and self-antigens. Analogous programs of sequential *Igh*, *Igk*, and *Igl* gene assembly drive B cell development in the adult bone marrow [1].

Thus, a single V(D)J recombinase must be directed to specific AgR loci, or even distinct regions within a given locus, by cell type-, developmental- and allele-specific regulatory mechanisms. Over the past 20 years, an overwhelming body of evidence has emerged to support what is now generally called the “accessibility model” for control of V(D)J recombination [21]. In brief, this model predicts that directed changes in chromatin associated with *Ig* and *Tcr* gene segments will determine their relative access to the V(D)J recombinase complex. To orchestrate this dynamic control, genetic elements that regulate chromatin accessibility at AgR loci rely on sophisticated crosstalk with epigenetic pathways, which will be the central focus of this chapter.

2. Epigenetic Regulation of Chromatin Accessibility

The genomic complement of eukaryotes is packaged into chromatin, which not only allows it to fit in a ~10 μ m nucleus but also creates opportunities for stringent regulation of gene expression. A key component of many gene regulatory networks is the modulation of chromatin compaction, which in turn controls the accessibility of associated DNA targets to nuclear factors important for transcription activation or repression [22,23]. Indeed, very early studies of eukaryotic chromatin revealed two broad levels of organization; euchromatin, which is relatively accessible to nuclear factors and harbors most active genes, and heterochromatin, which is largely inaccessible and contains silent genes [24]. The basic building blocks of chromatin are nucleosomes, which consist of DNA (~150 base pairs) wrapped around an octamer of four different histones (H2A, H2B H3 and H4). Stretches of nucleosomal DNA can adopt many configurations that differ in levels of compaction and, therefore, in their accessibility to nuclear factors.

A general mechanism for the dynamic regulation of chromatin involves covalent modification of nucleosome tails, which protrude from the core histone octamer [22,25]. A wide assortment of these epigenetic modifications can be stamped onto N-terminal tail residues of each histone, including lysine acetylation (Kac), lysine methylation with varying stoichiometries (Kme1, me2, and me3), arginine methylation (Rme), serine phosphorylation, and lysine ubiquitination. In addition, the DNA component of chromatin can be epigenetically modified by methylation of CpG dinucleotides, which normally tags a given region for gene silencing. Each of these marks is reversible, relying on a diverse set of “writers” (e.g., histone acetyl transferases, HATs) or erasers (e.g., histone deacetylases, HDACs) that can be chaperoned to a given gene by transcription factors [26].

It has now become clear that patterns of epigenetic modifications generate interaction surfaces for nuclear factors to alter chromatin accessibility and/or regulate transcription. In essence, each modification is a “letter” in the “epigenetic code” [25]. Because epigenetic modifications are reversible, their patterns can be revised rapidly in response to extracellular stimuli or developmental cues. The setting and re-setting of epigenetic patterns is at the heart of gene expression changes that drive development, permitting the use of a single genetic blueprint to generate the ~220 cell types in our bodies [27]. Indeed, the epigenetic code affects all aspects of DNA metabolism, including transcription, replication, repair, and recombination. A general model for how the epigenetic code is translated into gene activation or repression has emerged from correlative studies of histone modifications and the factors that target these marks. We will now describe a general model for gene activation, while reminding the reader that specifics of these processes vary from gene to gene and may even vary at a given gene depending on the stimulus.

Silent genes are characterized by a lack of acetylation marks on H3 and H4 tails, which are instead methylated at the K9 and K27 positions of H3 (H3K9me and H3K27me) [22,28]. Most histone modifiers lack DNA recognition motifs, and therefore rely on association with specific sets of transcription factors (TFs) for their targeting to genetic loci [23,29]. Upon transmission of the proper developmental/activation cues, TF-modifier complexes bind to cognate sites in gene regulatory elements (e.g., promoters and enhancers). In the case of gene activation, the specific set of modifiers recruited to cis elements usually includes erasers of repressive marks (H3K9 and K27 demethylases) and writers of activation marks. The latter set normally lead to acetylation of H3 and H4 at numerous positions by HATs and methylation of the H3K4 residue by histone methyltransferase complexes [22]. For example, active promoters become highly enriched for the tri-methyl mark on H3K4 via recruitment of the mixed-lineage leukemia (MLL) methyltransferase complex [30].

Revisions to a local epigenetic landscape trigger a cascade of events that ultimately lead to gene activation or repression. Upon receiving activation cues, acetylated histones on a given control region are recognized by bromodomain-containing proteins. This collection of nuclear factors includes nucleosome remodeling complexes, such as SWI-SNF, which utilizes ATP to generate more accessible chromatin configurations in the region [31,32]. Opening of regional chromatin may permit binding of additional factors to cis elements, including the TATA binding protein (TBP), which is an essential component of transcription initiation complexes at most promoters. Importantly, components of the general transcription machinery also recognize acetylated H3/H4 via bromodomains, which likely stabilize interactions between promoters and RNA Pol II-containing complexes [22,29]. Thus, gene activation (and repression) relies on a dynamic crosstalk between genetic elements and TF-histone modifier complexes, which revise local patterns of epigenetic marks and ultimately set the accessibility status of a chromatin domain.

3. Accessibility Control of V(D)J Recombination

Early evidence that chromatin accessibility plays a key role in controlling the tissue- and stage-specific aspects of AgR gene assembly derived from locus expression studies. Alt, Perry and colleagues reported a tight correlation between the transcription of unrearranged gene segments at *Ig* loci and their recombination potential [21,33]. For example, germline transcription of *Jk* gene segments occurs only at the pre-B cell stage of development, where they are targeted for rearrangement after a productive *Igh* allele is made [17,34]. Subsequent studies extended the correlations between germline transcription and recombination to all AgR loci. These observations produced the earliest version of the “accessibility model” since mechanisms that generate open chromatin for access to RNA polymerase may also establish a recombinase-accessible configuration at neighboring RSSs [21,35]. Perhaps the process of transcription itself might also enhance or propagate chromatin accessibility, a complex issue that remains only partially resolved (see below).

In the ensuing 20 years, support for the recombinase accessibility model came from numerous fronts [1,20,36]. In vitro studies clearly demonstrated that RSS cleavage by RAG-1/2 complexes is essentially blocked upon incorporation of a substrate into nucleosomes [37,38]. Cleavage of nucleosomal substrates is restored by a combination of treatments that mediate histone acetylation and remodeling by SWI-SNF [39]. Further support for the accessibility model derived from studies using intact cells, which showed that the stage- and tissue-specific recombination potential of AgR loci is recapitulated by its sensitivity to nucleases, including DNase, restriction enzymes, or recombinant RAG complexes [40–43]. More recently, direct evidence for the accessibility model was obtained from RAG chromatin immunoprecipitation assays (ChIPs), demonstrating that RAG binds to AgR gene segments with the predicted specificity [44]. For example, RAG complexes decorate the *Jk* cluster in pre-B but not in pro-B cells. Collectively, these findings indicate that chromatin accessibility is modulated during lymphocyte development to permit or deny recombinase access to specific clusters of gene segments.

In keeping with this model of recombination control, ChIP analyses have established numerous correlations between histone modifications, transcription, and V(D)J recombination [1,41,42, 45,46]. Pioneering studies by Krangel and colleagues showed that histone acetylation is a reliable metric for recombinase accessibility at *Tcr* loci during thymocyte development [46]. Similar studies by Sen at the *Igh* locus demonstrated that VH gene segments are hyperacetylated in pro-B cells (but not T lineage cells), when they are targeted for recombination [47]. Hyperacetylation of VH chromatin is at least partially erased upon differentiation to the pre-B cell stage, which presumably inhibits VH-to-DHJH recombination and helps enforce *Igh* allelic exclusion. In contrast to histone acetylation, H3K9me and K27me are marks for

recombinationally inert gene segments [48,49]. Our group established a causal relationship between histone modifications and recombinase accessibility using a well-characterized *Tcrb* mini-locus system. Specifically, we showed that recruitment of the H3K9 methyltransferase, G9a, to a recombinationally-competent substrate generated an inaccessible epigenetic landscape and, importantly, crippled germline transcription and D β -to-J β recombination [48]. Thus, recruitment of a single histone modifier can reverse the recombination potential of neighboring gene segments. However, identification of the relevant histone modification enzymes and the factors that recruit them to regulate AgR gene assembly in vivo remains an unsolved problem.

4. Topological Control of AgR Gene Assembly

Although directed changes in chromatin accessibility are critical for controlling AgR gene assembly, mounting evidence indicates that a number of additional strategies are employed by lymphocytes to ensure stage-, tissue-, and allele-specificity [1,20]. The most definitive evidence for these “beyond accessibility” control mechanisms came from Krangel and colleagues, who inserted a strong transcriptional enhancer (*E α*), which is specific for DP thymocytes, proximal to a V β gene segment. In wild-type *Tcrb* loci, transcription and recombination of the V β cluster is normally extinguished in DP cells to maintain allelic exclusion. Strikingly, the *E α* insertion led to inappropriate germline transcription of the neighboring V β gene segment in DP thymocytes, which also exhibited many hallmarks of accessible chromatin [50]. Despite its accessible configuration, the V β gene segment did not rearrange efficiently in DP cells.

Clues to how “beyond-accessibility” control is achieved were garnered from studies of gene regulation in diverse settings, which showed that gene expression relies on dynamic changes in chromosomal topology [51–53]. For example, fluorescence in situ hybridization (FISH) revealed that silent genes generally reside at the nuclear periphery, where association with nuclear lamina or sequestration away from TFs may lead to repression [53]. In contrast, active genes are centrally localized in the nucleus. The transcriptional status of some genes also correlates with co-localization to distinct chromosomal domains. Transcriptional silencing of the *Dnnt* gene is accompanied by its relocation to pericentromeric regions of heterochromatin [54]. Recent studies with more complex genetic loci, such as those encoding globins, cytokines, or antigen receptors, indicate that physical interactions between distant regulatory elements on the same or different chromosomes play a major role in the specificity of gene expression [55]. For example, the TH2 cytokine and interferon- γ loci, which reside on separate chromosomes, physically associate in mature T cells [56]. This interchromosomal association may maintain both genes in a state that is poised for expression following T cell activation.

Many of these topological processes are now known to correlate with the activation status of AgR loci during lymphocyte development. However, it should be stressed that functional relationships between topology and recombination potential still need to be established. Singh and colleagues first showed that *Igh* loci migrate from the nuclear periphery to a more central location during developmental progression from multi-potent lymphoid progenitors (*Igh* silent) to pro-B cells (*Igh* active) [57]. Strikingly, these studies also indicated that *Igh* concomitantly undergoes a dramatic conformational change, in which it converts from an extended to a highly compact configuration in pro-B cells [57]. Presumably this conformational change places the distant VH and DHJH clusters into spatial proximity, facilitating their recombination. Subsequent high-resolution FISH analyses demonstrated that the large VH cluster adopts a rosette configuration that establishes close contacts with the DHJH region [58]. Importantly, *Igh* compaction is reversed in pre-B cells, segregating the VH and DHJH clusters to help enforce allelic exclusion [59]. Similar conformational changes that correlate with rearrangement status have now been shown for nearly all *Ig* and *Tcr* loci [60,61].

Although these topological considerations are likely a key component of the AgR gene assembly program, the remainder of the chapter will focus on chromatin accessibility mechanisms. However, it is important to keep in mind that each of these regulatory strategies may be inextricably linked via contingent pathways. For example, locus compaction may first require chromatin opening over the VH cluster (or vice versa). Elucidation of such contingent pathways and their function in AgR gene assembly will be an important focus of the field in the coming years.

5. Genetic Elements That Control AgR Locus Accessibility

The historic links between V(D)J recombination and germline expression of gene segments hinted that transcriptional control elements might also be an integral genetic component of the AgR gene assembly program. In this regard, an important function of such cis elements, which include promoters and enhancers is to modulate the accessibility of neighboring chromatin to nuclear factors that either facilitate or repress transcription. Thus, it is a logical extrapolation that a similar set of genetic elements might simultaneously modulate access to the recombinase machinery. We now consider how the two best-characterized cis elements, promoters and enhancers, control V(D)J recombination via cross-talk with epigenetic pathways.

5.1 Enhancers

The classical definition of an enhancer is a genetic element that confers transcriptional competence to promoters over large distances and in an orientation-independent manner. From an epigenetic perspective, enhancers serve as conduits for TFs and their interacting histone modifiers, generating highly accessible or even nucleosome free regions in chromatin. Indeed, the extreme hypersensitivity of enhancers to DNase digestion became a powerful method to identify these elements in genetic loci [40,62]. Emerging epigenomic studies have shown that many tissue-specific enhancers also share a common epigenetic signature, consisting of histone hyperacetylation, association with the CBP/P300-HAT complex, high levels of H3K4me1, and only modest levels of H3K4me3 [63–65].

All seven AgR loci harbor at least one long-range enhancer element, which is usually situated at the 3' end of the locus near constant region coding exons [1]. Transgenic and knockout studies revealed a dominant role for enhancers in controlling AgR gene assembly. For example, deletion of the *Tcrb* enhancer, E β , abrogates germline transcription and recombination in thymocytes over the entire 25 kb region spanning both D β J β clusters (Fig. 1A). Consistent with a regulatory model involving enhancer-epigenetic crosstalk, E β deletion is accompanied by a loss of histone acetylation and an increase in H3K9me at D β J β clusters in thymocytes [41,66]. A connection between these properties and chromatin accessibility was established using restriction endonucleases, which are blocked from cognate sites in *Tcrb* upon enhancer deletion [41,42]. Similar findings at other AgR loci indicate that enhancers serve as long-range accessibility control elements (ACEs) for V(D)J recombination [1]. The remarkable range of ACE function is exemplified by the *Tcra* enhancer (E α), which is located 3' of the C α constant region in *Tcra/d*. Deletion of E α inhibits transcription and recombination of J α and V α segments at distances of up to 500 kb [67]. At *Igh*, removal of the intronic enhancer, E μ , significantly reduces recombination within the 20 kb DHJH cluster and completely blocks VH-to-DHJH assembly [68,69]. Recent studies by Sen and colleagues show that E μ enforces histone acetylation over a distance of up to 50 kb, spanning past the DHJH cluster [70]. Thus, enhancers appear to initiate the opening of chromatin at most AgRs through their function as long-range ACEs.

5.2 Promoters

Promoters direct transcription initiation by providing a genetic platform for the organization of transcription-competent Pol II complexes. Promoters are distributed throughout AgR loci, many of them proximal to individual gene segments or gene segment clusters [1]. In the germline configuration, active AgR promoters express sterile transcripts. However, upon productive V(D)J recombination, promoters associated with each V element drive transcription of the mature AgR gene. The vast majority of promoters in *Ig* and *Tcr* loci are enhancer-dependent, with modest inherent transcriptional activity. Collectively, these observations suggested that, in contrast to the long-range ACE function of enhancers, germline AgR promoters have more localized effects on chromatin accessibility, perhaps restricted to neighboring gene segments.

Support for a localized ACE function by promoters came from several approaches. Deletion of a *Tcrb* germline promoter that is located proximal to the D β 1 gene segment (PD β 1) inhibits transcription and recombination only at the neighboring D β 1J β cluster [71,72]; these processes are unperturbed at the downstream D β 2J β cluster, which has its own germline promoter (see Fig. 1) [73]. Corresponding losses in histone acetylation were observed at the D β 1 but not D β 2 cluster in PD β 1 knockout thymocytes, with the most prominent hypoacetylation restricted to a small D β 1-proximal region [42,72]. Further insights into PD β 1 function came from promoter-repositioning studies in mini-locus substrates. Unlike enhancer elements, which function at large distances, substrate recombination was exquisitely sensitive to the location of PD β 1. When positioned only several hundred bp downstream of the D β 1 gene segment, PD β 1 no longer supported D β -to-J β recombination despite retaining its ability to drive transcription through neighboring J β segments [74].

The relevance of these findings to endogenous *Tcrb* loci was confirmed in thymocytes from animals harboring a PD β 1 knockout. Deletion of this germline promoter has only modest effects on chromatin accessibility throughout the D β 1J β cluster (as judged by nuclease sensitivity), with the remarkable exception of a 200–300 bp region downstream of D β 1, which is highly resistant to nucleases [42]. This finding indicates that E β has an inherent long-range ACE function, conferring at least a partly open chromatin configuration over most of the D β J β cluster. However, the D β 1-proximal region is protected from this chromatin modulating activity of E β , requiring a highly localized ACE function of PD β 1 to unlock the D β 1 gene segment for germline transcription and recombination [42].

The function of promoters as local or regional ACEs appears to be a common regulatory theme within AgR loci. Deletion of the V β 14 promoter inhibits its germline transcription and recombination while having little effect on other V β gene segments [75]. However, the long-range ACEs that control V β promoters, if they exist, have yet to be identified. A more regional strategy is employed at the *Tcra* locus, where several germline promoters are sprinkled throughout the large *Ja* cluster [76,77]. The 5' promoter, termed TEA, is most active in thymocytes harboring germline *Tcra* loci, focusing initial recombination events to gene segments in this region [78]. Upon V α -to-*Ja* recombination, TEA is deleted and downstream promoters become more transcriptionally active, facilitating secondary rearrangements to these gene segment clusters [77]. The sequential recombination process enabled by this promoter gradient within the *Ja* cluster appears to be an important for generating a fully competent TCR α repertoire, by allowing multiple V α *Ja* combinations to pair with TCR β in each thymocyte [79].

One mechanism by which promoters function as localized ACEs has emerged from recent studies. A region within the RAG2 protein forms a motif called the plant homeodomain (PHD), which specifically recognizes the H3K4me3 mark in chromatin [80,81]. This modification is highly enriched near transcription initiation sites at promoters of actively transcribed genes

[82]. Indeed, ChIP analysis demonstrates that RAG2 is bound near active promoters throughout the genome rather than restricted to AgR loci [44]. Thus, targeting of recombinase activity likely relies on the binding of RAG1 to accessible RSSs, since RAG2 binding is more promiscuous. Notwithstanding, the high density of H3K4me3 on RSS chromatin near germline promoters may stabilize RAG-1/2 association, providing one aspect of localized ACE function [83]. Moreover, Lieber and colleagues showed that a distinct function of RAG2-H3K4me3 interactions is to enhance the enzymatic activity of RAG complexes [84]. Thus, binding of promoter-proximal chromatin by RAG2 may play several roles in targeting V(D)J recombination.

5.3 Promoter-Enhancer Holocomplexes

The split nature of promoter-enhancer architecture at AgR loci necessitates communication between these two elements over large distances in the genome. Several models to explain this through-space interaction have been proposed (reviewed in [51,85]); however the most common mechanism to emerge from molecular studies of β -globin and other loci is a direct promoter-enhancer bridge, forming a transcriptional holocomplex [86]. This bridge would generate a loop of intervening DNA and may allow an enhancer to deliver factors directly to promoters, facilitating the organization of competent transcription complexes [52,66].

Initial evidence that AgR promoters and enhancers form holocomplexes came from studies of the *Igk* locus. Using chromatin conformation capture (3C), Garrard and colleagues showed that distal *Igk* enhancers were positioned in spatial proximity to $\text{V}\kappa$ promoters in B cells lines harboring $\text{V}\kappa\text{J}\kappa$ rearrangements [87]. We have since examined promoter-enhancer communication during the initial stages of *Tcrb* activation in thymocytes. ChIP assays revealed that promoter-specific factors, such as SP1 and TBP, also cross-linked chromatin associated with E β , suggesting a spatial proximity between the two elements [42]. Importantly, deletion of PD β 1 abolished these interactions, discounting the possibility that SP1 and TBP are bound to cryptic sites in E β . The existence of a PD β 1-E β holocomplex was confirmed in primary DN thymocytes using 3C, which established that spatial proximity between E β and D β 1 regions was both enhancer- and promoter-dependent [42]. An intriguing possibility to explore in future studies is whether promoter-enhancer holocomplexes play any role in conformational changes at AgR loci that accompany lymphocyte development. One could envision that such holocomplexes at DJ clusters form one abutment in a bridge to distal V clusters, facilitating locus contraction.

In summary, known genetic control elements play distinct roles in guiding the ordered program of AgR gene assembly, as exemplified by a stepwise model for *Tcrb* activation (Fig. 1B). In DN thymocytes, the long-range ACE function of E β is activated by unknown mechanisms. This aspect of E β function spreads partially accessible chromatin throughout the D β J β clusters, with the exception of a privileged region surrounding D β 1. The partially accessible chromatin permits binding of additional factors to PD β 1, which form a holocomplex with E β . The PD β 1-E β holocomplex likely creates a unique interaction surface, which may allow recruitment of critical factors for the localized ACE function of PD β 1. Once fully engaged, PD β 1-ACE activity would free remaining chromatin barriers at the D β 1-RSS and initiate the *Tcrb* assembly program.

6. Chromatin Remodeling

An essential step in the activation of any gene is the remodeling of inaccessible chromatin to generate an open configuration. In eukaryotes, changes in chromatin configuration are largely mediated by a group of ATP-driven nucleosome remodeling complexes, of which SWI-SNF is the founding member [31]. SWI-SNF can enhance the accessibility of chromatinized DNA by several mechanisms, including removal or repositioning of nucleosomes [31,88].

Several lines of evidence suggest that SWI-SNF might be an important component of genetic-epigenetic crosstalk used to establish recombinase-accessible chromatin at AgR loci. First, SWI-SNF components have bromodomains, which may target them to acetylated histones on gene segment clusters near active promoter-enhancer complexes [32]. Second, RAG-dependent cleavage of nucleosomal substrates is stimulated by SWI-SNF in vitro [39]. Third, a catalytic component of SWI-SNF, termed BRG1, associates with recombinationally active regions within AgR loci in cell models [89]. Fourth, we showed that BRG1 is recruited to *Tcrb* in thymocytes and requires the presence of both ACEs (PD β 1 and E β) [90].

One potential role for SWI-SNF at *Tcrb* is the final “unlocking” of promoter-proximal D β 1 chromatin, exposing the RSS to recombinase (Fig. 1B). To test this possibility, we replaced PD β 1 in chromatinized substrates with Gal4 docking sites, which destroys their recombination potential. However, recruitment of a Gal4-SWI-SNF chimera to the promoterless substrate restored both germline transcription and recombination [90]. This finding indicates that the localized ACE function of PD β can be replaced completely by SWI-SNF and suggests that the “raison d’être” of PD β is SWI-SNF recruitment. This model is consistent with the loss of BRG1 binding to the D β 1J β cluster upon PD β 1 deletion [90].

Functional evidence for the importance of SWI-SNF in *Tcrb* chromatin accessibility was obtained using knockdown approaches. Depletion of SWI-SNF in DN cells severely impairs both germline transcription and recombination of D β 1J β clusters [90]. Thus, current evidence supports an activation model at *Tcrb*, in which PD β 1 forms a holocomplex with E β , allowing recruitment of SWI-SNF, which then unlocks chromatin associated with the D β 1-RSS. Consistent with this model, our more recent studies confirm that loss of BRG1 binding at *Tcrb* via deletion of either PD β 1 or E β increases nucleosome occupancy within the D β 1J β cluster [91].

The generality of these SWI-SNF-dependent mechanisms is supported by our recent studies of *Igh* regulation in pro-B cells. Analogous to *Tcrb*, the strong intronic enhancer, E μ , is required for tissue-specific recruitment of BRG1 to the DHJH cluster [92]. Depletion of SWI-SNF in pro-B cells dramatically attenuates all forms of transcription throughout this 2.5 Mb locus, including sense and anti-sense transcription of the large VH cluster (see below). Importantly, SWI-SNF deficiency also inhibits DH-to-JH as well as VH-to-DHJH recombination in pro-B cell models. Together, these findings suggest that SWI-SNF may play a universal role in generating recombinase-accessible chromatin throughout all seven antigen receptor loci. However, enhancer-promoter holocomplexes may focus its nucleosome remodeling activities to achieve more localized changes that unlock recalcitrant chromatin or sculpt AgR repertoires by creating nucleosome-free RSSs that are highly recombinogenic.

7. Transcription Factors as Liaisons for Genetic-Epigenetic Crosstalk

A critical relay between genetic control elements and epigenetic pathways is provided by TFs, which bind cis elements and often associate with histone modifiers. In addition, evidence is beginning to accumulate that TFs may facilitate bridging between distal control elements, generating new interaction surfaces to focus the activities of activation or repression complexes that contain distinct sets of nucleosome remodelers [93]. The role of TFs in regulating chromatin accessibility at AgR loci still remains a developing field. Classic genetic approaches have established the importance of specific factor binding sites for transcriptional control by promoter or enhancer elements. However, many of these studies were performed with reporter/transgene constructs or in transformed cells. When analogous mutations of TF sites were introduced into endogenous loci, it was often found that the loss of factor binding had only modest effects due to redundancies built into gene regulatory networks [94]. Similarly, approaches to determine the role of a given TF by either knocking it down or out can be difficult

to interpret due to the potential for indirect effects; e.g., the depleted factor regulates a second gene encoding a more relevant protein. Despite these complications, studies of TFs are moving forward, largely aided by the advent of ChIP, ChIP-chip, and ChIP-Seq methodologies, which afford invaluable information regarding the location of factor binding within a given locus or genome-wide [95–97].

To date, the strongest links between a TF and its involvement direct in controlling chromatin accessibility at AgR loci have been forged for the products of the lymphoid-restricted *Tcfe2a* gene, which encodes two highly related isoforms of helix-loop-helix proteins, called E12 and E47 [98,99]. These proteins bind to “E-boxes” found in a collection of lymphocyte genes, most notably in the cis elements controlling *Ig* and *Tcr* loci [99]. Over the years, it has become clear that E12/47 play numerous roles in both B and T cell biology, including its multi-faceted functions in activating several AgR loci. Early knockout studies of the *Tcfe2a* showed that it is required for both *Rag* gene expression and *Igh* recombination, the latter of which may be mechanistically tied to E-boxes present in $\text{E}\mu$ [100]. A more definitive knock-in approach was used to show the importance of E12/47 in *Igk* assembly. Mutation of the E-boxes in the intronic iEk enhancer severely impairs $\text{V}\kappa$ -to- $\text{J}\kappa$ rearrangement in pre-B cells, demonstrating a critical role for E12/47 in the ACE function of iEk [94]. Strikingly, a converse approach – overexpression of E12/47 – in a non-lymphoid cell line (human embryonic kidney, HEK) is sufficient to activate germline transcription and recombination (upon RAG-1/2 co-expression) of both *Ig* light chain loci [101]. E12/47-dependent activation of *Igk* and *Igl* in these cells is accompanied by increases in germline transcription over the V and J clusters targeted for efficient recombination [102].

Similarly, E12/47 appear to control many aspects of AgR gene assembly in thymocytes. *Tcfe2a* knockout mice exhibit perturbations in their γ/δ T cell repertoire due to aberrations in gene segment utilization [103]. In what may be a related finding, E12/47 overexpression induces a restricted subset of γ/δ gene segments for transcription/rearrangement in the non-lymphoid HEK cell system mentioned above [104]. More recent examination of *Tcfe2a* knockout mice also revealed its important role in driving efficient $\text{V}\beta$ -to- $\text{D}\beta\text{J}\beta$ recombination. Using a ChIP-chip approach, Murre and colleagues showed that E12/47 bind multiple $\text{V}\beta$ gene segments in DN thymocytes and are required for regional histone acetylation, supporting a functional link between E12/47 and $\text{V}\beta$ accessibility [95]. Moreover, E12/47 binding at the $\text{V}\beta$ segments is lost upon pre-TCR signaling, suggesting that diminished E12/47 activity during the DN to DP thymocyte transition may help enforce *Tcrb* allelic exclusion [95]. With regard to chromatin accessibility mechanisms, E12/47 interact directly with HATs and HAT-containing activation complexes, including P300/CBP and SAGA [95,99]. Thus, E12/47 may be more global liaisons between AgR cis elements and factors that regulate chromatin accessibility to V(D)J recombinase. Given the speed with which ChIP-based technologies are advancing, similar insights into the role of other TFs in controlling recombinase accessibility at *Ig/Tcr* loci are expected in the near future.

8. The Many Faces of Transcription in AgR Chromatin Accessibility

Ironically, the accessibility model emerged from early correlations between transcription and V(D)J recombination; however, the causal links between transcription and accessibility remain incomplete after more than two decades. Part of the difficulty in unraveling these links may be due to the numerous types of transcripts involved in activating large AgR loci (see below). Moreover, the role of transcription itself in opening chromatin may be distinct from the ACE function of cis elements that induce transcription. With regard to the latter possibility, conventional germline transcripts derived from PD β 1 appear to be dispensable for activation of neighboring $\text{D}\beta$ -to- $\text{J}\beta$ recombination. Instead, only the active promoter, not readthrough transcription, is required [74]. This finding is consistent with the localized ACE function of

PD β , which likely is needed only to form a holocomplex with E β and thereby recruit SWI-SNF for remodeling of the D β 1-RSS.

In contrast, transcriptional readthrough is essential for recombination of the large J α cluster. Krangel and colleagues showed that insertion of a transcriptional terminator at several locations within the cluster had no effect on the acetylation/recombination efficiencies of J α segments upstream of the terminator (actively transcribed) but dramatically impaired these metrics of recombinase accessibility at J α segments downstream of the block (no transcription) [105]. Thus, conventional germline transcription may be essential in larger gene segment clusters, where promoter-directed accessibility must be propagated over long distances. Consistent with this possibility, chromatin modifiers travel with active Pol II complexes, which may also aid in the spread of nucleosome remodeling machines throughout a transcribed locus [106]. In contrast, transcription may simply be a by-product of promoter function when only localized changes in accessibility are required (i.e., the shorter D β 1J β cluster).

In addition to classical germline transcripts, which derive from defined promoters in AgR loci and traverse gene segments in a “sense” direction, Corcoran and colleagues have shown that *Igh* is a template for several types of antisense transcripts [107,108]. These intergenic transcripts are pro-B cell-specific and apparently encompass large regions of the VH and DHJH clusters [108]. Although the origins of intergenic VH transcripts remain to be defined, DHJH anti-sense transcripts originate near the intronic enhancer and are dependent on both E μ and SWI-SNF [92,107]. Based on their stage-specific regulation, Corcoran has proposed that intergenic transcripts play a pioneering role in opening chromatin over large regions of *Igh* [107,108]. Although this hypothesis remains to be proven, intergenic transcription has been linked functionally to gene activation and chromatin opening at several other genetic loci, most notably β -globin [109–111]. Indeed, recent transcriptome analyses indicate that tissue-specific enhancers drive bi-directional transcription, which may be required for their gene regulatory functions [64].

A diametric role for intergenic transcription within the DH cluster has been proposed by Sen and colleagues. This group has shown that both sense and anti-sense transcription traverses the entire DH cluster [112]. The sense transcripts likely derive from cryptic promoters located near each of the 13 DH segments, and are E μ -dependent. All but the two flanking DH segments (Q52 and FL16.1) compose a long region of tandem repeats, likely due to gene duplication during evolution. Strikingly, the remaining 11 core DH segments reside in an extended region of inaccessible chromatin, which lacks histone hyperacetylation observed at the DQ52 and DFL16.1 “book-ends” [112]. Coincidental sense/anti-sense transcription is one hallmark of repeat-induced heterchromatin formation in many eukaryotes, which relies on RNA interference-mediated mechanisms [113]. Importantly, the chromatin status of DH gene segments correlates very well with their utilization in DHJH joins during B cell development [112]. As such, this DH repression mechanism may have evolved to normalize gene segment usage, avoiding an over-representation of the nearly identical, 11 core DHs, while the distinctive DQ52 and DFL16.1 segments are spared repression due to regional divergence during evolution. Collectively, studies of transcription and its role in generating recombinase-accessible or -inaccessible chromatin have revealed a remarkably diverse set of mechanisms that are relevant to local, regional, and long-range regulation at many genetic loci.

9. Concluding Remarks

Over the past two decades, our understanding of how crosstalk between genetic control elements and epigenetic pathways contribute to the “accessibility model” for AgR gene assembly has matured significantly. It is now clear that AgR locus opening is a highly ordered process, with the activation of long-range ACEs by lymphocyte-specific TFs mediating the

spread of accessible chromatin over large stretches of gene segments (Fig. 1B). Many of these long-range ACEs are enhancer elements that search out and eventually form stable complexes with promoters located proximal to target gene segments. In turn, promoter-enhancer holocomplexes focus the activity of epigenetic modifiers and nucleosome remodeling machines to augment accessibility both regionally and in a highly localized manner near promoters.

Some of the epigenetic changes resulting from promoter-enhancer crosstalk communicate directly with recombinase. In particular, the interaction between RAG2 and the promoter-rich H3K4me3 mark augments recombinase activity. Transcription and accompanying changes in the regional epigenetic landscape near promoter-enhancer holocomplexes clearly generate regional chromatin environments that permit recombinase access to proximal RSSs. As proposed by Schatz and Oettinger, the focused binding of RAG to such regions of highly accessible chromatin (e.g., the D β J β clusters) may generate recombination centers for the capture of distant gene segments that are distributed over much larger regions [44,83]. Future studies are needed to determine whether there is any relationship between these recombination centers and nuclear substructures that support robust transcription, termed transcription factories [55].

Further work is also required to decipher the precise mechanisms by which genetic control elements and their cognate transcription factors crosstalk with epigenetic pathways. For example, the factors involved in recruiting chromatin modifiers and remodeling complexes to cis elements at AgR loci remain largely unknown. Similarly, nuclear proteins that serve as the glue for promoter-enhancer holocomplexes need to be identified. Ultimately, the goal of such work will be to establish a real-time “motion picture” for each AgR locus, which will illuminate the order of factor recruitment, contingent changes in epigenetic landscapes, and the final emancipation of RSSs from chromatin, rendering them accessible to recombinase.

Relative to regional accessibility control, we currently know little about what regulates long-range recombination between V gene segments and their target (D)J partners. However, the advent of ChIP technologies, coupled with next-generation sequencing, should yield important clues in the near future. A particular focus will be the search for cis elements that facilitate locus contraction and the opening of chromatin over large V clusters. Indeed, several seemingly distinct approaches to decipher V segment usage, epigenetic and factor-binding landscapes, as well as high-resolution studies of locus topologies should soon coalesce to provide deeper insights into long-range mechanisms involving genetic-epigenetic crosstalk that are employed to sculpt immune repertoires. Moreover, such studies will contribute new paradigms for the chromosomal dynamics that guide AgR assembly and prevent aberrant interactions that lead to oncogenic translocations with other chromosomes.

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Figure 1. Model of genetic-epigenetic crosstalk and chromatin accessibility control at AgR loci
 (A) Basic genetic architecture of mouse *Igh* (top) and *Tcrb* (bottom), not drawn to scale. Variable (V, blue), diversity (D, red), and joining (J, green) gene segments as well as constant region exons (black) are represented as rectangles of the indicated colors. The purple rectangles depict relative positions of the E μ and E β enhancers. The lower panel shows a magnification of D β J β clusters within *Tcrb*. Black triangles represent RSSs. (B) Model for stepwise activation of D β J β clusters in pro-T cells. Panel (i) shows early stages of activation in DN thymocytes when only a subset of TFs binds to E β and PD β 1, activating the long-range ACE function of E β , which displays the enhancer-specific mark H3K4me1 (yellow triangles). Panel (ii) depicts the spread of partially open chromatin by E β as indicated by the H3/H4ac marks (orange circles), which permits additional TF binding at PD β 1. Panel (iii) shows PD β 1-E β holocomplex formation, which presumably allows interaction with additional factors, including SWI-SNF. In panel (iv) recruited SWI-SNF remodels neighboring D β 1 chromatin, allowing Pol II-directed transcription and rendering the D β 1-J β RSSs (black triangles) accessible to RAG-1/2 complexes. Germline transcription also deposits H3K4me3 marks (blue cups) near PD β 1, which stabilize local interactions with RAG2 via its PHD motif.