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Cis-Regulatory Elements and Epigenetic Changes Control Genomic Rearrangements of the IgH Locus

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Abstract

Immunoglobulin variable region exons are assembled from discontinuous variable (V), diversity (D), and joining (J) segments by the process of V(D)J recombination. V(D)J rearrangements of the immunoglobulin heavy chain (IgH) locus are tightly controlled in a tissue-specific, ordered and allele-specific manner by regulating accessibility of V, D, and J segments to the recombination activating gene proteins which are the specific components of the V(D)J recombinase. In this review we discuss recent advances and established models brought forward to explain the mechanisms underlying accessibility control of V(D)J recombination, including research on germline transcripts, spatial organization, and chromatin modifications of the immunoglobulin heavy chain (IgH) locus. Furthermore, we review the functions of well-described and potential new cis-regulatory elements with regard to processes such as V(D)J recombination, allelic exclusion, and IgH class switch recombination.

1. INTRODUCTION

An individual clone of mature B-cells expresses immunoglobulin (Ig) molecules as an antigen receptor. The typical sub-unit of an Ig molecule consists of two identical heavy chains (HC) and two identical light chains (LC). The N-terminal region of these chains contains the highly variable antigen binding site; whereas the C-terminal part is called constant region (C region). The C region of the IgH chain (C_H) determines the effector functions of antibodies, which are the secreted form of Ig molecules.

Immunoglobulin (Ig) and T-cell receptor (TCR) variable region exons are assembled from large arrays of V (variable), D (diversity), and J (joining) gene segments during the development, respectively, of B and T lymphocytes. Once a functional immunoglobulin chain is expressed, allelic exclusion operates through a feedback mechanism to prevent further rearrangements of Ig heavy (IgH) and Ig light (IgL) chain genes. V(D)J recombination is mediated by a common recombinase complex that includes the recombination-activating gene products RAG1 and RAG2, which harbor endonuclease activity that introduces DNA double strand breaks (DSBs) at V, D, and J segments. The V(D)J reaction is completed by the ubiquitously expressed nonhomologous end-joining (NHEJ) factors that join the broken V, D, and J segments together. Still, Ig loci are only fully assembled in B lineage cells and TCR loci are only assembled in T lineage cells. Within a lineage, different loci are rearranged in a specific order. For example, IgH locus variable region exons are assembled before those of Ig light chains (IgL), and within the IgH locus D to J_H recombination precedes V_H to DJ_H recombination. Given such locus-specific regulation and a common V(D)J recombinase, accessibility of the different loci to the common V(D)J recombinase must underlie the cell-type and stage-dependent assembly of the different IgH and TCR gene families (Jung *et al.*, 2006).

Activation of mature B-cells can alter their IgH loci through a separate form of genomic rearrangement which is termed IgH class switch recombination (CSR). CSR allows B-cells to express IgH chains with different constant regions which can change the effector functions of antibodies without altering variable region specificity. CSR is initiated by activation-induced cytosine deaminase (AID), the activity of which ultimately leads to DSBs in regions upstream of C_H genes which are then joined by NHEJ or other end-joining pathways to complete the CSR reaction (Chaudhuri *et al.*, 2007).

Ig and TCR loci contain a number of cis-regulatory elements which regulate V(D)J rearrangements, IgH CSR, and Ig gene expression at various levels. In this review, we will focus on the impact of cis-regulatory elements on genetic and epigenetic regulation of recombination events within the IgH locus.

2. THE IMMUNOGLOBULIN HEAVY CHAIN LOCUS

The murine IgH locus is a complex genomic region, spanning about 3 Mb close to the telomere of the long arm on chromosome 12. The IgH locus comprises arrays of V, D, and J segments upstream of several constant region exons (Fig. 1.1A). Different mouse strains carry varying numbers of V_H and D elements. Some 150 V_H segments are distributed over ~2.5 Mb in the 5′ part of the IgH locus and are classified in 16 V_H gene families defined by sequence similarities (Johnston *et al.*, 2006). These V_H gene families are partially interspersed with one another but, depending on position, can be divided into proximal (3′ part of the V_H cluster, close to IgH–D region, for example, V_H7183), intermediate (e.g., V_HS107), and distal (5′ part of the V_H cluster, distant from IgH–D region, for example, V_HJ558) families. 3′ of the V_H elements, separated by ~90 kb, lie 10–15 D segments (Retter *et al.*, 2007; Ye, 2004) followed by 4 J_H elements. Because to the uniform transcriptional orientation of V, D, and J segments, V(D)J recombination events at the IgH locus result in deletion of the intervening sequence. The 3′ part of the IgH locus contains a series of sets of different constant (C) region exons Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, Cα, which will be referred to as "C_H genes" (Fig. 1.1B).

A large number of cis-regulatory elements were identified throughout the IgH locus. The intronic enhancer, Eμ, is located in the intron between J_H4 and the C_H exons (Fig. 1.1); the 3′ IgH regulatory region (IgH 3′RR) consists of several DNase hypersensitive sites and is located at the very 3′ end of the IgH locus (Fig. 1.1). Transcriptional promoters are present upstream of every V_H segment (Fig. 1.2B), upstream of D_H segments (Fig. 1.2A and C), and upstream of C_H genes (Fig. 1.2A). In addition, antisense transcripts from less well-defined promoters were described in the V_H, D_H, J_H regions, and upstream of C_H genes. Section 7 of this chapter contains a detailed discussion of these IgH cis-regulatory elements.

3. V(D)J RECOMBINATION DURING B-CELL DEVELOPMENT

The IgH locus V(D)J exon is assembled at the pro-B-cell stage leading to the production of μ IgH heavy chains via splicing of the V_HDJ_H exon onto the adjacent Cμ constant region exons. Functional μHC and surrogate Ig light chain proteins form a complex that is expressed on the surface of pre-B-cells and is known as the pre-B-cell receptor (pre-BCR) (Cobb *et al.*, 2006). Signaling through the pre-BCR induces proliferation, signals cessation of further V_H to DJ_H rearrangements at the IgH locus (i.e., allelic exclusion, see below), and promotes the onset of IgL variable region exon (V_LJ_L) assembly. Thus, expression of the pre-BCR represents an important checkpoint at the pro- to pre-B-cell transition (Mårtensson *et al.*, 2007). Subsequently, Igκ and Igλ LC variable regions are assembled during the pre-B-cell stage. Expression of a functional Igκ or Igλ LC along with μHC forms a complete Ig molecule which is expressed on the cell surface of the resulting immature B-cells (Gorman and Alt, 1998). Immature B-cells migrate to the periphery where mature naïve B-cells can

be activated and undergo further modification of their IgH locus including IgH CSR and somatic hypermutation (SHM) (see below).

All V, D, and J segments are flanked by recombination signal sequences (RSSs) that consist of a conserved palindromic heptamer and a conserved AT-rich nonamer separated by a less conserved 12 bp or 23 bp spacer (Sakano *et al.*, 1980). The RAG1/2 endonuclease recognizes and binds a pair of RSSs with different spacer lengths in the context of the 12/23 rule (Early *et al.*, 1980; Sakano *et al.*, 1980), which allows for efficient V(D)J recombination only between gene segments flanked by 12 bp and 23 bp RSSs (Fugmann *et al.*, 2000). The 12/23 restriction provides some direction for which Ig gene segments can be assembled. For example, IgH D segments are flanked with 12 bp RSSs on both sides; whereas V_H and J_H segments are flanked with 23 bp RSSs, thus preventing direct V_H–J_H joining. In the TCRβ locus, however, direct Vβ to Jβ joints would be allowed according to the 12/23 rule but are denied by “beyond 12/23” restrictions (Bassing *et al.*, 2000). Differential composition of RSSs implement “beyond 12/23” restriction at the nicking and pairing step of V(D)J recombination (Drejer-Teel *et al.*, 2007; Jung *et al.*, 2003).

RAG cutting precisely between RSSs and variable region gene segments results in formation of blunt RSS ends, and the formation of coding ends (CE) of the V, D, or J segments as closed hairpins. Coding joints (CJs) are formed through a joining reaction mediated by members of the NHEJ repair pathway. In this reaction, Ku proteins bind the free CEs and recruit DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which activates the endonuclease activity of Artemis to open the hairpins. Subsequently, ends are joined by the XRCC4/DNA ligaseIV complex (Rooney *et al.*, 2004). In contrast, the blunt SEs are precisely ligated to each other by NHEJ.

Tight regulation of V(D)J recombination is imperative to ensure proper lymphocyte development and genomic integrity. While V(D)J recombination is of enormous advantage in order to efficiently combat infections, erroneous V(D)J recombination can have adverse consequences including chromosomal translocations, which can contribute to neoplastic transformation and the development of leukemias and lymphomas.

4. CLASS SWITCH RECOMBINATION AND SOMATIC HYPERMUTATION

After a V_HDJ_H variable exon is assembled upstream of the C region exons, a promoter 5′ of the rearranged V_H segment drives expression of μ and δ HC molecules in mature B-cells. Upon antigen encounter and activation, a B-cell can switch to expression of downstream C_H genes to generate antibodies with the same variable region specificity but a different C_H effector function by CSR. Repetitive switch (S) regions are located upstream of every C_H gene except Cδ. Introduction of DSBs in Sμ and a downstream switch region can result in joining of the two switch regions, deletion of the intervening sequence, and consequently expression of a downstream C_H gene with the same variable region exon. AID is absolutely required for CSR. It appears to function by deaminating cytosines to uracils within the substrate S region DNA with the resulting mismatches somehow being processed into DSBs by cooption of normal repair pathways (Di Noia and Neuberger, 2007). As AID is a single-strand DNA-specific cytosine deaminase, its activity on duplex S region DNA is targeted by transcription (Chaudhuri *et al.*, 2007). In this context, switch regions can be transcribed from an I (intervening) promoter located upstream of each S region, which allows for AID targeting to specific transcribed S regions (Fig. 1.2B). In addition to these sense germline transcripts, antisense transcripts were described in several S regions (Apel *et al.*, 1992; Julius *et al.*, 1988; Morrison *et al.*, 1998; Perlot *et al.*, 2008). AID initiated S region DSBs are joined by NHEJ or an alternative end-joining pathway to complete CSR.

AID is also required for SHM, a process during which the variable region exon gets mutated at a relatively high frequency in activated B-cells. SHM is initiated by transcription-dependent targeting of AID to assembled variable regions followed by error prone repair of the resulting mismatches (Di Noia and Neuberger, 2007). Through affinity maturation, B-cell clones producing higher affinity antibodies are selected and an efficient adaptive immune response is elicited.

5. IgH REARRANGEMENTS AND ALLELIC EXCLUSION

Expression of RAG1 and RAG2 is absolutely required for V(D)J recombination. In the haematopoietic lineage, RAG activity can first be demonstrated in common lymphoid progenitor (CLP) cells, which are precursor cells that can develop into B-cells, T-cells, natural killer (NK) cells, and dendritic cells (DC) (Borghesi *et al.*, 2004). Together with the detection of D to J_H rearrangements in non-B-cell lymphoid lineages (Borghesi *et al.*, 2004; Born *et al.*, 1988; Kurosawa *et al.*, 1981), expression of RAG in CLPs suggests that the first IgH rearrangement step can occur, at least at low level, in CLPs. Thus, the IgH D to J_H recombination step is not absolutely restricted to the B-lineage, in contrast to V_H to DJ_H rearrangements which normally occur only in B-cells. Efficient D to J_H rearrangement on both IgH alleles takes place after B lineage commitment in the pro-B-cell stage (Alt *et al.*, 1984).

Once DJ_H segments are formed, one of the upstream V_H elements can join to form a complete V_HDJ_H exon. In the murine IgH locus, proximal V_H segments are preferentially rearranged compared to distal V_H elements throughout ontogeny (Malynn *et al.*, 1990; Yancopoulos *et al.*, 1984). However, peripheral B-cells do not show this preference as selection alters the B-cell repertoire (Yancopoulos *et al.*, 1988). Both D to J_H and V_H to DJ_H recombination take place at the pro-B-cell stage, however, in an ordered manner such that D to J_H rearrangement nearly always occurs before V_H to DJ_H rearrangement (Alt *et al.*, 1984). In this regard, V_H to DJ_H recombination is the step that is regulated in the context of allelic exclusion to ensure expression of only one functional HC.

Successful V_H to DJ_H recombination and expression of a μHC from one IgH allele, prevents a second DJ_H allele from undergoing V_H to DJ_H rearrangement (Jung *et al.*, 2006). Considering the junctional diversity generated during V(D)J recombination, only one out of three V_HDJ_H exons will be in frame with the downstream Cμ exons; whereas two out of three will be out of frame and therefore unable to express a functional μHC (Mostoslavsky *et al.*, 2004). The percentage of functional recombination events is further decreased by usage of V_H pseudogenes containing stop codons, frameshifts, defective splice sites, or lacking an ATG translation start site, by stop codons in D_H segments as well as through selection against certain reading frames of DJ_H joins (Gu *et al.*, 1991). As a result, a substantial fraction of developing B-cells will not be able to generate a functional μHC from either IgH allele and will undergo apoptosis (Rajewsky, 1996). If a nonfunctional V_H to DJ_H rearrangement occurs on the first allele, the second DJ_H allele can still undergo V_H to DJ_H rearrangement (Alt *et al.*, 1984).

Allelic exclusion of V_H to DJ_H rearrangement is mediated by feedback regulation; a functional μHC together with surrogate light chains are assembled to a pre-BCR, which signals the cessation of further V_H to DJ_H rearrangements (Alt *et al.*, 1984; Jung *et al.*, 2006). In this regard, endogenous IgH rearrangements are largely inhibited by the expression of a preassembled membrane-bound μHC transgene (Nussenzweig *et al.*, 1988). Likewise, allelic exclusion was broken by targeted deletion of the μHC transmembrane exons (Kitamura and Rajewsky, 1992), by lack of a functional pre-BCR (Löffert *et al.*, 1996), and by combined deletion of the downstream pre-BCR signaling molecules Syk and

ZAP-70 (Schweighoffer *et al.*, 2003). The combined data from these studies strongly support a feedback-mediated mechanism for allelic exclusion that is mediated by signaling through a functional μHC in the pre-BCR signaling complex.

The complete chain of events that leads to cessation of V_H to DJ_H rearrangements and implementation of allelic exclusion is still elusive. However, it was shown that the onset of allelic exclusion after successful V_H to DJ_H recombination is accompanied by the transient downregulation of RAG (Grawunder *et al.*, 1995), decontraction of the IgH locus (Roldán *et al.*, 2005), and loss of accessibility correlates such as V_H germline transcripts and marks of active chromatin (see below). It has been estimated that only 1 in 10,000 wild-type B-lymphocytes actually escape allelic exclusion and express a functional μHC from both IgH alleles (Barreto and Cumano, 2000). Feedback regulation can explain cessation of V_H to DJ_H rearrangement but would be ineffective if both IgH alleles would rearrange simultaneously. Therefore, it was suggested that the V(D)J recombination machinery targets one allele at a time (Alt *et al.*, 1980). Supportive of this hypothesis was the observation that all Ig loci as well as the TCRβ locus undergo asynchronous replication (Mostoslavsky *et al.*, 2001; Norio *et al.*, 2005). At the allelically excluded Igκ locus it is thought that asynchronous replication facilitates allele specific chromatin changes (Mostoslavsky *et al.*, 1998) that lead to the early replicating allele rearranging first (Mostoslavsky *et al.*, 2001). A similar mechanism for V_H to DJ_H recombination, the allelically excluded IgH rearrangement step, was speculated, but has not yet been demonstrated. Thus, asynchronous replication could conceivably play a role in the initiation phase of allelic exclusion. However, it does not provide an explanation for the maintenance of allelic exclusion during subsequent B-cell stages, which prevents further IgH rearrangements in the presence of RAG, which must be effected by feedback mechanisms that influence accessibility.

6. ACCESSIBILITY CONTROL

The accessibility hypothesis was proposed to explain how a single common V(D)J recombinase can target the different Ig and TCR loci in a lineage- and stage-specific manner (Yancopoulos and Alt, 1985). For example, Ig variable region exons are only fully assembled in B-cells while TCR variable region exons are only rearranged in T-cells. Similarly, IgH loci are rearranged during the pro-B-cell stage and not in pre-B-cells where IgL variable region assembly occurs. The accessibility hypothesis was first proposed based on the finding that germline V_H gene segments are transcribed in pro-B-cells but not in subsequent B-cell stages, with germline V_H transcription providing a potential correlate of accessibility (Yancopoulos and Alt, 1985). This hypothesis was proven by experiments that showed transfected TCR gene substrates could be rearranged by pro-B lines that do not rearrange endogenous TCR gene segments, first demonstrating a common V(D)J recombinase (Yancoupouls, 1986). This conclusion was confirmed and extended by other studies (Krangel, 2003; Stanhope-Baker *et al.*, 1996). However, the precise mechanisms that mediate differential accessibility of Ig and TCR gene segments to V(D)J recombination are still not clear. Over the decades, several correlates of accessibility have been defined and a general picture is beginning to emerge as to how accessibility control might be regulated and implemented. Among the known correlates of accessibility are germline transcripts, chromatin modifications, DNase hypersensitivity, spatial organization, and positioning of Ig and TCR loci in the interphase nucleus.

6.1. Germline transcripts

Germline transcription is the production of transcripts from V, D, or J segments and adjacent regions before they undergo rearrangement (Fig. 1.2). Sense germline transcripts starting from promoters upstream of V, D, and J segments have been described in all Ig and TCR loci (Hesslein and Schatz, 2001), and their stage-specific expression patterns strongly

correlate with accessibility of these transcribed elements (e.g., Yancopoulos and Alt, 1985). The precise role of sense germline transcripts is still not understood and has been debated (Krangel, 2003). Recent studies support the notion that active transcription mediates chromatin changes that render the transcribed regions accessible to the recombinase (Sen and Oltz, 2006). However, it has been debated whether germline transcripts are the cause or the effect of these chromatin changes, and neither possibility has been unequivocally proven or disproved. On one hand, the levels of germline transcripts exhibit a positive correlation with rearrangement efficiency (Sun and Storb, 2001), which could suggest that the process of transcription itself could promote RAG targeting. However, others have shown that the correlation between individual rearrangements and germline transcription is not absolute (Angelin-Duclos and Calame, 1998; Sikes *et al.*, 2002).

The IgH locus in germline configuration is transcribed from the promoter of DQ52 (PDQ52), the 3′ most D_H segment, towards Cμ, thereby producing the so-called μ0 transcript (Fig. 1.2A). After D to J_H rearrangement, the recombined DJ_H element is transcribed (Fig. 1.2C) (Alessandrini and Desiderio, 1991; Reth and Alt, 1984); and at the same time, unrearranged V_H segments are transcribed from their promoters (Fig. 1.2B). Germline V_H transcription appears to be silenced upon a productive rearrangement (Corcoran, 2005; Yancopoulos *et al.*, 1985).

More recently, antisense transcripts have been found to occur throughout the V_H cluster (Fig. 1.2B) (Bolland *et al.*, 2004), in the D_H region (Fig. 1.2A and C) (Bolland *et al.*, 2007; Chakraborty *et al.*, 2007), and in the J_H region (Fig. 1.2A) (Bolland *et al.*, 2007; Perlot *et al.*, 2008). V_H antisense transcripts appear to be biallelic, and it has been argued that such transcripts are large and span several V_H segments and the adjacent intergenic regions; but formal proof of their initiation sites is still lacking. V_H antisense transcription was shown to initiate during D to J_H recombination, and to be rapidly downregulated after V_H to DJ_H recombination (Bolland *et al.*, 2004). D_H antisense transcripts were detected in RAG-deficient pro-B-cells as well as on the D–J_H rearranged allele of B-cell lines with a functionally assembled IgH gene (Chakraborty *et al.*, 2007). D_H antisense transcripts have been suggested to originate from the 3′ most D_H (Chakraborty *et al.*, 2007) or from the J_H region (Bolland *et al.*, 2007).

The functional significance of antisense transcription in the context of V(D)J recombination has not been fully elucidated. It has been postulated that antisense transcription promotes an active chromatin state rendering the locus more accessible (Bolland *et al.*, 2004), based on the observed correlation between antisense V_H germline transcription and active V_H to DJ_H recombination (Bolland *et al.*, 2004). Similar conclusions were reached based on the observation of reduced antisense D_H transcripts and reduced D to J_H rearrangements in mice lacking the intronic enhancer, Eμ (Afshar *et al.*, 2006; Bolland *et al.*, 2007). Conversely, others have raised the possibility that antisense transcripts, at least in the DSP D_H segments, could pair with low levels of postulated germline sense transcripts and elicit RNA interference-mediated transcriptional gene silencing (Chakraborty *et al.*, 2007; Koralov *et al.*, 2008). It should be noted that true germline sense transcripts have not been identified as yet in the germline D_H segments, but their level may be as low as those originally identified in the *S. pombe* centromeric repeats and may only be revealed in an RNAi-deficient background (Volpe *et al.*, 2002).

6.2. Spatial organization and nuclear positioning of the IgH locus

The spatial organization of the Ig and TCR loci was analyzed by three-dimensional fluorescence *in situ* hybridization (3D FISH), in which nuclear organization remains preserved. Several groups showed that before undergoing rearrangement, the IgH locus moves from its default position at the nuclear periphery to a more central compartment

(Fuxa *et al.*, 2004; Kosak *et al.*, 2002), going along with the observation that the nuclear periphery has a repressive effect on transcription (Andrulis *et al.*, 1998; Baxter *et al.*, 2002; Reddy *et al.*, 2008) and, therefore, might keep the IgH locus in an inaccessible state. These observations are consistent with the peripheral location of the IgH locus in thymocytes which have only low level of D to J_H and no V_H to DJ_H rearrangements (Fuxa *et al.*, 2004; Kosak *et al.*, 2002; Kurosawa *et al.*, 1981). The centrally located IgH locus in pro-B-cells can undergo D to J_H rearrangement; however, for rearrangements of the distant V_H elements, long-range contraction and looping of the IgH locus (Jhunjhunwala *et al.*, 2008) seems to be crucial as lack of IgH locus contraction in Pax5-deficient pro-B-cells does not allow for rearrangements of intermediate and distal V_H families (Fuxa *et al.*, 2004; Sayegh *et al.*, 2005). After successful rearrangement, the IgH locus decontracts and, thereby, has been proposed to impede further V_H to DJ_H rearrangements by increasing the distance between V_H elements and the DJ_H region (Roldán *et al.*, 2005). Therefore, it seems that one aspect of V_H to DJ_H recombination accessibility might be influenced by spatial arrangement of the IgH locus within the nucleus.

In B lineage stages subsequent to the pro-B stage, one IgH allele is positioned in close proximity to centromeric heterochromatin (Roldán *et al.*, 2005; Skok *et al.*, 2001). This finding was interpreted as the monoallelic silencing of the nonproductive IgH allele, because transcriptionally silent genes have been shown to associate with centromeric heterochromatin (Brown *et al.*, 1997). However, the V_H cluster gets silenced on both alleles in the context of germline transcription, and considering the fact that rearrangements from both IgH alleles, productive and nonproductive in either DJ_H or V_HDJ_H configuration, are expressed in all B-cell stages that were examined (Daly *et al.*, 2007; Fukita *et al.*, 1998; Ono and Nose, 2007), monoallelic silencing might be either a short-time transient phenomenon or recruitment to centromeric heterochromatin might have other implications in the process of allelic exclusion.

Studies of interactions between IgH and Igκ alleles demonstrated coordinated patterns of action of Ig loci during B-cell development. Interactions between IgH and Igκ, predominantly in pre-B-cells, were demonstrated to reposition the interacting IgH allele to centromeric heterochromatin and induce IgH locus decompaction (Hewitt *et al.*, 2008). Therefore, this interchromosomal interaction could play a role in IgH allelic exclusion and the transition from accessible IgH alleles to accessible Igκ alleles.

6.3. Chromatin modifications

Eukaryotic DNA is packaged into nucleosomes in which genomic DNA is wrapped around histone octamers. The N-terminal ends of histones, called histone tails, can be marked by diverse modifications (e.g., acetylation, methylation, phosphorylation, ubiquitination, and others). The “histone code” (Jenuwein and Allis, 2001) translates patterns of histone modifications into repression or activation of chromatin. An extensive effort has been made to investigate the effects of histone modifications and also various other chromatin attributes such as DNA methylation, DNase sensitivity, and nucleosome remodeling on accessibility of Ig and TCR loci with hopes of shedding light on the epigenetic regulation of V(D)J recombination.

Posttranslational modifications of N-terminal histone tails can affect genome regulation in several ways. Histone modifications can directly affect chromatin structure, for example, through a change in charge. In this context, histone acetylation can loosen the association between DNA and the histone core or can alter higher order chromatin packaging. Alternatively chromatin modifications can disrupt or provide binding sites for chromatin remodeling complexes or other effector molecules. Prominent examples of such specialized binding domains are bromodomains specifically binding acetylated lysines, and

chromodomains binding to dimethylated lysine 9 on histone 3 (Kouzarides and Berger, 2007).

Many studies showed that marks of active chromatin correlate with V(D)J rearrangements. For example, acetylated lysine 9 on histone 3 (H3K9ac), hyperacetylated histone 4, and dimethylated lysine 4 on histone 3 (H3K4me2) are active chromatin marks (Kouzarides and Berger, 2007). They are present in the D–J_H region peaking around the 5′ most D segment, DFL16.1, and over the J_H elements (Chakraborty *et al.*, 2007; Morshead *et al.*, 2003) in early pro-B-cells that are poised to undergo D to J_H rearrangements. However, they are almost absent in thymocytes (Chakraborty *et al.*, 2007). Following D to J_H recombination, the proximal V_H elements become hyperacetylated and, thereafter, in a manner that is dependent on IL-7R signaling and on its downstream effector STAT5 (signal transducer and activator of transcription 5), the distal V_H segments become hyperacetylated (Bertolino *et al.*, 2005; Chowdhury and Sen, 2001). Acetylation patterns seem to be narrowly confined to the V_H segment, its promoter, and RSS (Johnson *et al.*, 2003).

Histone hyperacetylation is lost after productive V_H to DJ_H recombination, thereby contributing to rendering the V_H cluster inaccessible in pre-B-cells (Chowdhury and Sen, 2003). Notably, an engineered locus that actively recruits an H3K9 methyltransferase shows downregulation of germline transcripts and impaired V(D)J recombination (Osipovich *et al.*, 2004). H3K9me2 is absent in the D–J_H region of pro-B-cells and present in thymocytes (Chakraborty *et al.*, 2007), and removal of H3K9me2 from the V_H region before V_H to DJ_H recombination is dependent on Pax5 (Johnson *et al.*, 2004), a transcription factor essential for B-cell commitment (Busslinger, 2004). In agreement with this data, loss of Pax5 leads to an inability to rearrange distal V_H gene families (Hesslein *et al.*, 2003; Nutt *et al.*, 1997).

The antagonistic Polycomb (PcG) and Trithorax (trxG) groups of protein complexes establish and propagate a silenced or active chromatin state, respectively (Ringrose and Paro, 2004). Curiously, targeted deletion of the PcG protein Ezh2, an H3K27 methyltransferase, inhibits rearrangements of the distal V_HJ558 family without affecting germline transcription (Su *et al.*, 2003). H3K27 methylation was reported to be a mark of inactive chromatin (Kouzarides and Berger, 2007); therefore, it remains to be determined whether the results observed in the Ezh2 knock out are direct or indirect effects.

Recent studies reported that the PhD finger domain of RAG2 specifically binds to trimethylated H3K4 (Liu *et al.*, 2007; Matthews *et al.*, 2007), a histone modification associated with transcriptional start regions (Pokholok *et al.*, 2005) also shown to be present in accessible IgH regions (Liu *et al.*, 2007). Mutation of the conserved tryptophan residue W453 within the PhD finger domain of RAG2 abrogates RAG2 binding to H3K4me3 and impairs V(D)J recombination of chromosomal and extra-chromosomal substrates (Liu *et al.*, 2007; Matthews *et al.*, 2007). However, removal of the entire RAG2 noncore region, including the PhD domain, only leads to a partial impairment of V(D)J recombination (Liang *et al.*, 2002). These seemingly contradicting data have been suggested to reflect the presence of an inhibitory function within the noncore region of RAG-2, which is relieved upon binding to H3K4me3, or can be circumvented by deleting the entire noncore region (Liu *et al.*, 2007; Matthews *et al.*, 2007). These recent studies provide the first direct link between epigenetic control of V(D)J rearrangement and RAG recombinase accessibility.

Chromatin remodeling complexes can change the composition, structure, or position of nucleosomes within chromatin. These changes are noncovalent and are dependent on ATP hydrolysis (Martens and Winston, 2003). The SWI/SNF chromatin remodeling complex contains a bromodomain that allows it to efficiently bind acetylated chromatin and mobilize nucleosomes or change nucleosome structure (Martens and Winston, 2003). In this regard, it

was shown that unmodified or even hyperacetylated nucleosomes located directly on RSSs are inhibitory to RAG cleavage *in vitro* (Golding *et al.*, 1999) and that addition of SWI/SNF improved substrate cleavage (Kwon *et al.*, 2000). RSSs strongly attract nucleosomes and, thus, may implement some aspect of accessibility control (Baumann *et al.*, 2003). Moreover, nucleosome positioning appears pivotal for V(D)J recombination *in vivo* (Cherry and Baltimore, 1999). Further supporting the importance of SWI/SNF complexes in V(D)J recombination, BRG1 (the ATPase subunit of SWI/SNF) was found to associate at Ig and TCR loci within hyperacetylated chromatin regions (Morshead *et al.*, 2003). Functional targeting of BRG1 to a TCRβ minilocus lacking the essential Dβ promoter rescued V(D)J recombination of this substrate (Osipovich *et al.*, 2007), substantiating the role of SWI/SNF complexes in V(D)J recombination and suggesting a role for transcriptional promoters in recruitment of chromatin remodeling complexes.

Another readout to assess chromatin structure is the DNase sensitivity assay. Less tightly packed chromatin- or nucleosome-free DNA is more sensitive to DNase or restriction enzyme digestion than heterochromatin regions. While cis-acting elements such as promoters and enhancers can be devoid of nucleosomes and, therefore, are DNase hypersensitive, accessible chromatin of Ig and TCR loci shows general DNase sensitivity (Yancopoulos *et al.*, 1986). In this context, the region between DQ52 and Eμ is DNase sensitive before D to J_H rearrangement and J_H RSSs show enhanced sensitivity and seem to be nucleosome-free (Maës *et al.*, 2006). The V_H region becomes nuclease sensitive before V_H to DJ_H rearrangement and reverts to a refractory state after successful V_H to DJ_H recombination (Chowdhury and Sen, 2003).

Cytosines in mammalian DNA can be methylated in CpG dinucleotides. Generally, cytosine methylation corresponds to silenced genes (Stein *et al.*, 1982; Vardimon *et al.*, 1982) or silent regions throughout the genome; whereas promoter regions of expressed genes are found in an unmethylated state. Cytosine methylation can act by inhibiting regulatory proteins from binding to DNA (Watt and Molloy, 1988), or by recruiting methyl-CpG binding proteins which in turn can interact with HDACs to enforce a silent chromatin state (Jaenisch and Bird, 2003). In this regard, methylated V(D)J recombination substrates are refractory to active rearrangement (Cherry and Baltimore, 1999; Hsieh and Lieber, 1992); in particular, methylated RSSs can abolish RAG cleavage and V(D)J recombination (Whitehurst *et al.*, 2000). Demethylation alone, however, is not sufficient to initiate V(D)J recombination (Cherry *et al.*, 2000). The D_H–J_H cluster gets demethylated before the onset of D to J_H recombination (Maës *et al.*, 2001; Storb and Arp, 1983) characteristic of an accessible state. In this context, the JCκ region gets monoallelically demethylated and this demethylated allele undergoes rearrangement first (Mostoslavsky *et al.*, 1998). The second allele stays in a repressive environment and somehow can get demethylated, if the rearrangement on the first Igκ allele is nonproductive (Goldmit and Bergman, 2004). More extensive studies on DNA methylation of the IgH locus could potentially help to elucidate aspects of accessibility control within this locus.

7. IgH LOCUS CONTROL THROUGH CIS-REGULATORY ELEMENTS

A formidable number of cis-regulatory elements have been identified throughout the IgH locus (Fig. 1.1). Enhancers are located in the J_H–Cμ intronic region and at the very 3′ end of the locus. Promoters are found 5′ of V_H and D segments as well as 5′ of most C_H genes. Cis-elements in the IgH locus not only govern gene expression, but also play crucial roles in accessibility control in all its above-mentioned aspects and also control CSR. An extensive effort has been made to elucidate the many roles of these transcription elements. Ongoing research also aims at identifying missing regulatory elements and elucidating their role in IgH locus control.

7.1. Promoter of DQ52

DQ52 is the 3′ most D segment. Its promoter becomes active before D to J_H rearrangement to generate the μ0 transcript (Fig. 1.2A) (Alessandrini and Desiderio, 1991; Kottmann *et al.*, 1994; Schlissel *et al.*, 1991a). This transcript runs all the way through the Cμ exons, which get spliced to the J_H1 splice donor site (Schlissel *et al.*, 1991b). The same promoter region also gives rise to a low-level antisense transcript (Chakraborty *et al.*, 2007). It has been suggested that the repetitive nature of the D_H region in combination with bidirectional transcription can elicit RNA interference-mediated transcriptional gene silencing that would lead to the observed inactive chromatin state of the DSP elements. However, as mentioned above, only antisense and no sense transcripts have been detected thus far in the germline D_H region (Chakraborty *et al.*, 2007).

Every D_H element upstream of DQ52 has a bidirectional promoter which, upon D to J_H rearrangement, potentially through approximation to the Eμ enhancer, gets activated to generate an antisense transcript and a sense transcript (Fig. 1.2C) (Alessandrini and Desiderio, 1991; Chakraborty *et al.*, 2007). The sense transcript gets spliced in a way that the rearranged DJ_H segment is joined to the Cμ exons. In one reading frame this mRNA can encode for a shorter version of the μHC (Reth and Alt, 1984), which can inhibit subsequent V_H to DJ_H rearrangements (Gu *et al.*, 1991; Löffert *et al.*, 1996; Malynn *et al.*, 2002). Targeted deletion of the DQ52 promoter, which has both promoter and enhancer activity (Kottmann *et al.*, 1994), in mice had no major impact on D to J_H rearrangement, other than a slight shift in J_H usage (Afshar *et al.*, 2006; Nitschke *et al.*, 2001). However, in these studies, μ0-like transcripts were still evident, suggesting that activity of the heterogeneous promoter of DQ52 was not entirely abrogated. In a different study, the intronic Eμ enhancer was replaced with a phosphoglycerate kinase promoter–neomycin resistance gene cassette ($PGK\text{-}Neo^R$), which resulted in complete absence of μ0 transcripts and complete inhibition of D to J_H rearrangement (Perlot *et al.*, 2005). In this regard, targeted deletion of an analogous promoter element in the TCRβ locus, the promoter of Dβ1, led to diminished germline transcripts from this promoter and reduced Dβ1 rearrangements (Whitehurst *et al.*, 1999), demonstrating an accessibility control function for this element in D_β–J_β recombination.

7.2. V_H promoters

Every V_H element has its own promoter that initiates V_H germline transcripts before V_H to DJ_H rearrangement (Fig. 1.2B), as well as transcripts of the assembled V_HDJ_H exon after rearrangement (Fig. 1.2D). Most V_H promoters can generate a germline transcript, in which a leader exon gets spliced to a V_H exon (Fig. 1.2B). The transcript gets polyadenylated and contains an open reading frame (Yancopoulos and Alt, 1985); however, no V_H protein or its function has thus far been demonstrated. The most conserved element across V_H promoters is the octamer ATGCAAAT (Parslow *et al.*, 1984). This sequence element has been shown to be necessary for V_H transcription (Mason *et al.*, 1985), and it binds the ubiquitously expressed Oct-1 and the B-cell-specific Oct-2, both POU family transcription factors. Most but not all V_H promoters contain a TATA box, an initiator (Inr) element (Buchanan *et al.*, 1997), a heptamer, and a pyrimidine stretch (Eaton and Calame, 1987). Additionally, binding sites for a number of mostly B-lineage-specific transcription factors and chromatin remodeling complexes have been identified in V_H promoter regions (Johnston *et al.*, 2006).

Germline transcripts from unrearranged V_H promoters are generated upon D to J_H rearrangement in pro-B-cells, and downregulated after completed V_H to DJ_H recombination and assembly and expression of a functional V_HDJ_H exon (Bolland *et al.*, 2004; Hardy *et al.*, 1991). The promoter of a recombined V_H element stays active throughout B-cell development, and cell line experiments showed that the first upstream unrearranged V_H

segment can also be continuously expressed at reduced levels (Wang and Calame, 1985). Promoter activity of a functionally rearranged V_H element was shown to be partially dependent on the 3′ regulatory region (Pinaud *et al.*, 2001). Thus, V_H promoters might fulfill a dual role: to help confer accessibility to germline V_H segments and to drive expression of the assembled heavy chain gene.

7.3. Intronic enhancer

The IgH intronic enhancer (Eμ) was the first cellular (as opposed to viral) eukaryotic enhancer element described (Alt *et al.*, 1982; Banerji *et al.*, 1983; Gillies *et al.*, 1983). Eμ comprises a 220 bp enhancer core (cEμ) and two flanking matrix attachment regions (MARs). Targeted deletion of both MARs shows that they are dispensable for efficient V(D)J recombination within the IgH locus (Sakai *et al.*, 1999). Deletion of Eμ in B-cells (Chen *et al.*, 1993; Sakai *et al.*, 1999; Serwe and Sablitzky, 1993) and in the germline of mice (Afshar *et al.*, 2006; Perlot *et al.*, 2005) led to reduced D to J_H rearrangement and severely impaired V_H to DJ_H rearrangement. The residual V(D)J recombination activity in the IgH locus implies that activation of IgH rearrangements may also involve one or more additional enhancer type elements. One candidate for such a compensating element is the promoter/enhancer region PDQ52, which was speculated to promote D to J_H recombination (Alessandrini and Desiderio, 1991). However, deletion of PDQ52 along with Eμ did not show increased impairment above that seen with deletion of Eμ alone (Afshar *et al.*, 2006). However, since the deletion of PDQ52 appeared to be incomplete, this element can not yet be ruled out as having redundant functions with Eμ in conferring accessibility to the D_H–J_H region. Another candidate for cooperative function with Eμ is the 3′ IgH regulatory region, but the double knockout of Eμ and the IgH 3′RR has not been generated. By analogy, deletion of the intronic Igκ enhancer (iEκ) reduces Vk to Jκ rearrangements (Xu *et al.*, 1996); whereas a double knockout of iEκ and the 3′Eκ enhancer completely blocks recombination of the Igκ locus (Inlay *et al.*, 2002). The iEκ and 3′Eκ in the Igκ locus are the enhancer elements corresponding to the position of Eμ and IgH 3′RR in the IgH locus.

It has been puzzling why in Eμ knockout mice the V_H to DJ_H step is more severely impaired than the D to J_H step, even though the Eμ enhancer has no obvious effect on germline transcripts of intermediate and distal V_H families (Perlot *et al.*, 2005). One explanation could be significant underestimation of D to J_H impairment in Eμ knockout mice. Initial very low levels of D to J_H rearrangements could limit the crucial DJ_H substrates for subsequent V_H to DJ_H rearrangements and, therefore, result in the observed strong reduction of V_H to DJ_H recombination. After a productive rearrangement, feedback regulation inhibits further V_H to DJ_H recombination, but does not block further D to J_H rearrangements (Reth *et al.*, 1987). Therefore, D to J_H recombination might “catch up” over the course of B-cell development and mask a stronger impairment.

Notably, replacement of Eμ with a PGK-Neo^R cassette (Chen *et al.*, 1993; Perlot *et al.*, 2005; Sakai *et al.*, 1999) or introduction of PGK-Neo^R cassette just 5′ of Eμ (Chen *et al.*, 1993; Delpy *et al.*, 2002) results in a much more severe impairment or a complete block of V(D)J recombination and concomitant complete loss of μ0 transcripts (Perlot *et al.*, 2005). This phenomenon could be explained by a promoter competition/insulating mechanism. In such a scenario, the PGK-Neo^R gene and its promoter might compete with PDQ52 for activity from a downstream cis-element such as the IgH 3′RR, which is known to act over long distances (Pinaud *et al.*, 2001). Similar promoter competition for the IgH 3′RR has been observed between I promoters and the PGK-Neo^R cassette introduced in the C_H region (see below). Alternatively, the PGK-Neo^R cassette could induce local chromatin changes that impede μ0 germline transcription and accessibility of D and J_H segments.

Extensive studies revealed an array of binding sites for B-lineage-specific transcription factors and also for ubiquitously expressed proteins within the Eμ enhancer and the flanking MARs (Calame and Sen, 2004). The unique combination of these factors is likely to mediate the enhancer's predominant activity in pro-B-cells (Inlay *et al.*, 2006). In this context, replacement of iEκ with Eμ leads to premature Igκ rearrangement in pro-B-cells and absence of Igκ rearrangements in pre-B-cells, the stage when LC rearrangement normally takes place (Inlay *et al.*, 2006), corroborating the pro-B-cell specificity of Eμ.

Eμ was suggested to play a role in regulating antisense transcripts through the J_H and D_H region (Afshar *et al.*, 2006; Bolland *et al.*, 2007), and additionally a promoter region within Eμ was identified that gives rise to the Iμ transcript (Lennon and Perry, 1985; Su and Kadesch, 1990). Starting at Eμ, this transcript extends through the μ switch region and Cμ. Transcription of switch regions was shown to be necessary for CSR, probably for targeting AID, and in this regard, deletion of Eμ leads to reduced Iμ transcript levels and reduced CSR (Bottaro *et al.*, 1998; Perlot *et al.*, 2005). Deletion of Eμ has no obvious effect on somatic hypermutation of V_HDJ_H exons in mature B-cells (Perlot *et al.*, 2005). An open question is how the activity of AID is specifically targeted to regions within Ig loci. It was speculated that cis-regulatory elements could determine this specificity, but neither cEμ nor the IgH 3′RR, alone (Morvan *et al.*, 2003), seem to have a crucial role in targeting SHM to the IgH locus V_HDJ_H segments. While an absolute requirement of cEμ or the IgH 3′RR for SHM can be excluded, there is a possibility that smaller defects of SHM in these mutants are masked by selection processes during affinity maturation. Also, a combined function of cEμ and the IgH 3′RR in promoting or targeting SHM is another possibility that needs to be tested.

7.4. 3′ IgH regulatory region and I promoters

I promoters are located upstream of all switch regions (Chaudhuri *et al.*, 2007; Lennon and Perry, 1985; Lutzker and Alt, 1988). Transcripts initiating from I promoters are processed in such a way that an I (intervening) exon, located immediately downstream of the I promoter, is spliced to the associated C_H exons. In this process, the intronic region including the S region is spliced out and the transcript gets polyadenylated. However, these transcripts appear "sterile," as they do not contain an open reading frame and could not be shown to encode for a protein (Chaudhuri *et al.*, 2007). Active transcription from I promoters is necessary for CSR as only transcribed S regions can become AID targets during CSR. In this context, deletion of I promoters abrogates efficient CSR to the associated C_H genes; while replacement of I promoters with a constitutively active promoter directs CSR to the associated C_H gene (Manis *et al.*, 2002). Transcription from different I promoters prior to CSR can be induced upon stimulation with different activators or cytokines. Corresponding surface receptors for these molecules and their associated downstream signaling pathways effect different combinations of activating or repressive response elements within I promoter regions, which leads to CSR to different IgH isotypes under different stimulation conditions (Stavnezer, 2000). Most I promoters do not appear to act in isolation as efficient transcription from them also requires the IgH 3′RR (Pinaud *et al.*, 2001) and physical interaction between the IgH 3′RR and specific I promoters has been implicated (Wuerffel *et al.*, 2007).

The IgH 3′RR is located downstream of Cα at the very 3′ end of the IgH locus (Fig. 1.2B). This regulatory region consists of a number of DNaseI hypersensitive sites scattered over ~35 kb (Dariavach *et al.*, 1991; Garrett *et al.*, 2005; Lieberson *et al.*, 1991; Matthias and Baltimore, 1993; Pettersson *et al.*, 1990); up until now, none of them were shown to play a role in V(D)J recombination but more studies are needed (Khamlichi *et al.*, 2000; Pinaud *et al.*, 2001). The most striking function, control of IgH CSR, has been assigned to HS3b, HS4 within the IgH 3′RR. Targeted deletions in mice revealed severely reduced CSR to most IgH isotypes and reduced germline transcription from I promoters through the corresponding S

regions (Pinaud *et al.*, 2001), a process required for CSR (Jung *et al.*, 1993; Zhang *et al.*, 1993). Deletion of the more 5′ DNaseI hypersensitive sites within the IgH 3′RR HS3a and HS1,2 had no effect on CSR (Manis *et al.*, 1998); however, replacement of HS3a or HS1,2 with a PGK-Neo^R cassette resulted in a similar defect as in the HS3b, HS4 deletion (Cogné *et al.*, 1994, Manis *et al.*, 1998). The latter observations suggested a potential promoter competition/insulation between I promoters and the PGK-Neo^R cassette for signals from within the IgH 3′RR. This hypothesis was strengthened by insertion of a PGK-Neo^R cassette at the Iγ2b promoter or the Cε gene, respectively. In both cases, germline transcription and class switching to C_H genes 3′ of the inserted PGK-Neo^R cassette was unaffected; while germline transcription and class switching to C_H genes 5′ of the inserted PGK-Neo^R cassette was impaired (Seidl *et al.*, 1999). These results suggest that the inserted PGK-Neo^R cassette can interfere with the long-range control effect of IgH 3′RR on CSR in a position-dependent manner.

The IgH 3′RR is necessary for efficient expression of the rearranged HC from the promoter upstream of the assembled V_HDJ_H exon (Pinaud *et al.*, 2001), whereas the much more proximal Eμ enhancer is not required for HC expression (Perlot *et al.*, 2005). Because it can influence expression of rearranged V_HDJ_H segments, the IgH 3′ RR can function over a distance of at least 200 kb. Such long-range activity may be important for activating oncogenes translocated into the upstream portions of the C_H locus in lymphomas. Not all of the seven described hypersensitivity sites in the spacious 3′ regulatory region have been knocked out yet, therefore, other potential functions still remain to be discovered. Apart from the above-mentioned effects on germline transcription, CSR, and IgH expression, it has been speculated that parts of the 3′ regulatory region might have a role in long-range chromatin organization. Finally, activity of the Iγ1 promoter does not appear to be dependent on the IgH 3′RR; suggesting that it carries sufficient regulatory elements itself or that there are other long range IgH locus elements that function in CSR to be defined.

7.5. Additional potential regulatory elements

Several laboratories suggested that the IgH locus can be associated with the nuclear periphery via its 5′ region (Kosak *et al.*, 2002; Yang *et al.*, 2005). The 5′ end of the IgH locus does not get deleted in the course of V(D)J recombination and as such is an attractive location for a missing regulatory element that controls processes such as accessibility control of the distal V_H genes, positioning of the IgH locus, or feedback regulation. In fact, ~30 kb upstream of the most distal V_H element an array of DNaseI hypersensitive sites has been identified (Pawlitzky *et al.*, 2006). One of these sites, HS1, was reported to be pro-B-cell specific and potentially contain binding sites for the transcription factors PU.1, Pax5, and E2A. However, preliminary knockout experiments, in which HS1 was deleted, showed no effect on the IgH locus, as targeted alleles could still undergo efficient V(D)J recombination including all V_H gene families. Furthermore, allelic exclusion was unaffected (Perlot, Pawlitzky, Brodeur, and Alt, unpublished data). Other potential functions of these sites, including acting as a boundary area as was suggested by DNA modifications confined to one side of 5′IgH hypersensitive sites (Reddy *et al.*, 2008), are still being tested.

Another area that was speculated to harbor a regulatory element is the ~90 kb region between the V_H and the D_H clusters. This region could contain an element that ensures the ordered rearrangement of the D to J_H and V_H to DJ_H steps, such as a boundary element that influences activation of separate IgH locus domains. Moreover, the V_H to D_H intergenic region is deleted on a productively rearranged allele but remains in place on a DJ_H rearranged allele, suggesting an element might reside in this region that is responsible for shutting down the incompletely rearranged allele in the context of allelic exclusion. Potential support for such an element came from placement of a V_H segment into the D_H region, which resulted in breaking of lineage specificity, ordered rearrangement, and allelic

exclusion of the introduced V_H segment (Bates *et al.*, 2007). Preliminary studies in which this intergenic region has been deleted have provided direct support for the notion that this region contains elements important for regulation of lineage specificity of V_H to DJ_H rearrangement (Giallourakis, Franklin, and Alt, unpublished data).

7.6. Interplay between cis-regulatory elements

The transition from an inactive to an active chromatin state of the IgH locus is in part governed by Eμ. The intronic enhancer plays an important role in placing active chromatin marks throughout the D_H–J_H region (Chakraborty, Perlot, Subrahmanyam, Alt, and Sen, unpublished data). In addition, Eμ promotes transcripts from PDQ52 and supports formation of the DNaseI hypersensitive site at this promoter element (Perlot *et al.*, 2005; Chakraborty, Perlot, Subrahmanyam, Alt, and Sen, unpublished data). These data argue in favor of a direct interaction between Eμ and PDQ52 reminiscent of a corresponding promoter/enhancer holocomplex described in the TCRβ locus (Oestreich *et al.*, 2006). The fact that Eμ is not absolutely required for stimulation of PDQ52 suggests partial compensation by another cis-element. It was speculated that the IgH 3′RR can take over this role of interaction with PDQ52 and of activating the D_H–J_H region, because its ability to function over a long range to activate I region promoters and to influence expression of the promoter of a rearranged V_HDJ_H segment (Pinaud *et al.*, 2001).

No single cis-regulatory element has thus far been identified that is responsible for the bulk of V_H germline transcription. It seems to be likely that V_H promoters can be activated in trans by B-lineage and stage-specific factors. V_H antisense transcripts might be a prerequisite of V_H sense germline transcripts by initializing an active chromatin state and accessibility of the V_H locus (Bolland *et al.*, 2004). Start sites of these transcripts still remain elusive, which exacerbates the manipulation of such transcripts and a direct proof for this hypothesis.

After rearrangement of a complete V_HDJ_H exon, the assembled V_HDJ_H–Cμ gene is transcribed from the V_H promoter 5′ of the rearranged V_H. Transcription from the rearranged V_H promoter is mainly supported by the IgH 3′RR (Pinaud *et al.*, 2001) suggesting direct interaction of the two cis-elements. In this regard, the ability of the IgH 3′RR to form a direct complex with a region around Eμ and downstream I promoters was demonstrated during CSR (Wuerffel *et al.*, 2007). However, cEμ does not appear to be involved in that interaction, since deletion of that element does not affect complex formation (Wuerffel *et al.*, 2007). As mentioned above, the interaction of the IgH 3′RR with I region promoters likely underlies the cooperation of these two elements in I region transcription and regulation of IgH CSR.

8. CONCLUSIONS

Antigen receptor genes are assembled by the process of V(D)J recombination in a developmentally controlled manner. Differential accessibility at Ig and TCR loci is regulated at least in part by cooperative action of cis-regulatory elements. Tremendous progress has been made in identifying and elucidating the multiple layers of control of the IgH locus during V(D)J recombination; however, many important questions remain unanswered and new questions are emerging. Among these are processes involved in ordered IgH rearrangements, asynchronous V_H to DJ_H rearrangements, enforcement of feedback regulation, the precise relevance and impact of chromosome positioning and movements, the role of antisense transcription throughout the IgH locus, and chromatin modifications. Likewise, a remarkable amount of progress has been made in elucidating the role of cis-acting elements in the regulation of IgH CSR, but again there are still many unanswered questions including precisely how these elements function to specifically target AID to S

regions and the precise mechanisms by which the IgH 3′RR and I region promoters elements cooperate in response to external stimuli to specifically activate CSR to particular C_H genes. To fully understand the genetic and epigenetic regulation of the IgH locus, all involved cis-regulatory elements and trans acting factors need to be identified and analyzed. More work also will need to be done to understand how these factors influence regulation at the level of chromatin structure and spatial organization. Understanding the mechanisms governing the IgH locus, a model system for gene expression and epigenetic regulation will also advance our understanding of various other unsolved biological problems.

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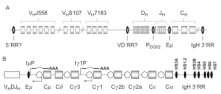

FIGURE 1.1.

Schematic depiction of the murine IgH locus. (A) V_H, D_H, J_H gene segments and C_H exons are shown as rectangles, known and potential regulatory elements as ovals. The V_H families V_HJ558, V_HS107, and V_H7183 are depicted as examples for distal, intermediate, and proximal V_H families, respectively. The cis-regulatory elements P_{DQ52} (promoter of DQ52), Eμ (intronic enhancer), and IgH 3′RR (IgH 3′ regulatory region) are depicted. The potential regulatory elements 5′RR (5′ regulatory region) and VD RR (V_H–D_H intergenic regulatory region) are depicted with a question mark. Drawing not to scale. (B) The 3′ part of the IgH locus. An assembled V_HDJ_H exon is shown as a white rectangle, C_H genes as squares, Eμ and individual DNaseI hypersensitive sites within the IgH 3′RR are depicted as black ovals, switch regions as white circles. I promoters are located upstream of every switch region (Chaudhuri *et al.*, 2007; Lennon and Perry, 1985; Lutzker and Alt, 1988), only μ and γ1 I promoters (IμP, Iγ1P) are depicted. Transcripts from I promoters get spliced and polyadenylated. Switch regions also get transcribed in the antisense orientation (Apel *et al.*, 1992; Julius *et al.*, 1988, Morrison *et al.*, 1998; Perlot *et al.*, 2008). Concomitant transcription from IμP and, for example, Iγ1P can target AID to μ and γ1 switch regions and thereby initiate CSR to Cγ1.

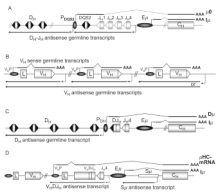

FIGURE 1.2.

Transcripts within the IgH locus. V_H, D_H, J_H gene segments and C_H exons are shown as rectangles, enhancer and promoter elements as ovals. 12 bp and 23 bp RSSs are depicted as black and white triangles, respectively. Drawings not to scale. (A) The IgH locus in germline configuration is transcribed from the promoter of DQ52 (P_{DQ52}) to produce the μ0 transcript (Alessandrini and Desiderio, 1991), and from within the Eμ enhancer to generate the Iμ transcript (Lennon and Perry, 1985; Su and Kadesch, 1990), both of which are getting spliced and polyadenylated (Kottmann *et al.*, 1994, Su and Kadesch, 1990). D_H and J_H elements are transcribed in the antisense orientation (Bolland *et al.*, 2007; Chakraborty *et al.*, 2007), suggested start sites (dashed arrows) are located around P_{DQ52} (Chakraborty *et al.*, 2007) and Eμ (Bolland *et al.*, 2007). Sites of transcriptional termination of D_H–J_H antisense germline transcripts are unknown. (B) Unrearranged V_H segments are transcribed in the sense orientation from the individual V_H promoters (V_HP) (Yancopoulos and Alt, 1985). The intron between the leader (L) and the V_H exon (V_H) is spliced out, and the V_H sense germline transcript gets polyadenylated (Yancopoulos and Alt, 1985). The V_H segments and V_H intergenic regions can also get transcribed in the antisense orientation (Bolland *et al.*, 2004). Start and termination sites of V_H antisense germline transcripts are unknown. Therefore, individual antisense transcripts could comprise one V_H segment and its adjacent regions or multiple V_H segments including intergenic regions, shown as short and long solid arrows, respectively. (C) Upon D to J_H recombination, the assembled DJ_H exon gets transcribed from the D_H promoter (P_{DH}) and spliced to the Cμ exons to generate the Dμ transcript (Alessandrini and Desiderio, 1991; Reth and Alt, 1984), which in one reading frame encodes for a short μHC molecule (Reth and Alt, 1984). D_H antisense germline transcription is present throughout the remaining unrearranged D_H segments (Chakraborty *et al.*, 2007). Suggested origin of D_H antisense germline transcripts is the region around the promoter of the recombined D_H segment (depicted as P_{DH}) (Chakraborty *et al.*, 2007), transcriptional termination sites are unknown. (D) Upon V_H to DJ_H recombination, the promoter of the rearranged V_H segment (depicted as V_HP) drives expression of mRNA encoding for the μHC. In addition to Iμ sense transcription, the Sμ switch region is also transcribed in the antisense orientation (Perlot *et al.*, 2008) from promoters residing within Sμ (Apel *et al.*, 1992; Morrison *et al.*, 1998), the transcriptional termination site of the Sμ antisense transcript is unknown. The assembled V_HDJ_H exon and the adjacent J_H region are transcribed in the antisense orientation potentially from start sites within the J_H cluster (dashed arrow) (Perlot *et al.*, 2008), the transcriptional termination site of the V_HDJ_H antisense transcript is unknown. Upstream unrearranged V_H segments are transcriptionally silenced upon assembly of a functional V_HDJ_H exon (Bolland *et al.*, 2004; Yancopoulos and Alt, 1985).