CHAPTER 2

Long Noncoding RNAs: Implications for Antigen Receptor Diversification

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Contents

Abstract

Noncoding RNAs (ncRNAs), both small and large, have recently risen to prominence as surprisingly versatile regulators of gene expression. In fact, eukaryotic transcriptomes are rife with RNAs that do not code for protein, though the

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majority of these species remains wholly uncharacterized. The functional diversity among the mere handful of validated ncRNAs hints at the vast regulatory potential of these silent biomolecules. Though the act of noncoding transcription and the resultant ncRNAs do not directly produce proteins, they represent powerful means of gene control. Here we survey the accumulating literature on the myriad functions of long ncRNAs and emphasize one curious case of noncoding transcription at antigen receptor loci in lymphocytes.

1. INTRODUCTION

The central dogma of biology—DNA makes RNA makes protein—summarizes one of the fundamental tenets of modern science (Crick, 1958, 1970; Watson, 1965). This skeletal outline, however, hardly captures the complex interplay between the biomolecular trio of life. Thus our understanding of gene expression has continued to evolve in order to account for this complexity. Much of gene function can be attributed to protein, which is understandably more chemically diverse (and by inference, more functionally diverse) than either of its nucleic acid predecessors. However, an astonishing breadth of RNA function has been revealed over the last several decades. In accordance with the central dogma, messenger RNA (mRNA) does indeed function as an intermediary between the DNA code and its final incarnation as protein. These messages, however, participate actively during gene expression, as they undergo editing, alternative splicing, and other co- and posttranscriptional modifications that substantially affect the quality and quantity of the encoded proteins.

The untranslated portions of mRNAs exert complex control over the stability and translation of their attached messages, hinting at the full functional capacity of RNA. These silent bits of RNA harbor docking sites for regulatory proteins, binding sequences for regulatory RNAs, or secondary fold motifs that respond to environmental changes. Bacteria are particularly adept at the latter strategy, using thermosensitive RNA motifs that permit translation *in cis* upon melting (Johansson *et al.*, 2002; Morita *et al.*, 1999; Nocker *et al.*, 2001) or RNA aptamers (riboswitches) that directly bind to metabolites to induce conformational changes that affect mRNA behavior *in cis* (Epshtein *et al.*, 2003; Mandal *et al.*, 2003; Mandal *et al.*, 2004; Sudarsan *et al.*, 2003; Winkler *et al.*, 2002a,b, 2003). In addition to transmitting environmental cues through mRNAs, noncoding RNA structures can function as independent catalytic units (Forster and Symons, 1987a,b; Guerrier-Takada *et al.*, 1983; Kruger *et al.*, 1982; Kuo

et al., 1988; Nielsen *et al.*, 2005; Salehi-Ashtiani *et al.*, 2006; Saville and Collins, 1990; Teixeira *et al.*, 2004). Embedded within a coding context, noncoding RNA domains exhibit many signs of complex behavior bordering on independence from proteins.

2. NONCODING RNAs

2.1. Infrastructural noncoding RNAs

Indeed, the information-bearing mRNAs are accompanied by a diverse collection of wholly noncoding RNAs (ncRNAs), demarcating a functional detour of some RNAs from the route between DNA and protein. Many of these ncRNAs participate in essential housekeeping functions, often as cogs within ribonucleoprotein (RNP) machines. Well-characterized RNP complexes that contain ncRNAs form the fundamental building blocks of the gene expression infrastructure. The spliceosome carries small nuclear RNAs (snRNAs) that direct mRNA splicing (Black *et al.*, 1985; Bringmann *et al.*, 1984; Chabot *et al.*, 1985; Lerner *et al.*, 1980; Rogers and Wall, 1980). Subsequent translation of these messages relies on decoding by ribosomal RNA (rRNA) and transfer RNA (tRNA) in the context of the ribosome. Together, these ncRNA-containing machines transport genomic information to its final destination. Participation of noncoding RNA in the gene expression workflow does not end there. Yet another RNP—the signal recognition particle (SRP)—targets certain nascent polypeptides to the endoplasmic reticulum in preparation for secretion or membrane insertion (Walter and Blobel, 1982). The noncoding RNA component of the SRP stimulates the catalytic properties of the particle and is essential for the process of protein recognition and translocation (Bradshaw *et al.*, 2009).

Chromosome integrity has also proven to depend, in part, on ncRNAs, particularly with regard to two distinct structural domains: the telomere and the centromere. Eukaryotes employ a clever RNA-dependent solution to circumvent the catastrophic shortening of linear chromosomes during replication—dubbed the "end replication problem." The enzyme telomerase elongates and maintains telomeres using an internal ncRNA component to template the reverse transcription of telomeric repeats (Shippen-Lentz and Blackburn, 1990). Centromeres are compacted as silent heterochromatin, the presence of which is essential for the binding of protein factors that regulate sister chromatid adhesion (Bernard *et al.*, 2001; Nonaka *et al.*, 2002). In fission yeast, the maintenance of this heterochromatic state depends on noncoding transcripts generated from centromeric repeats, giving rise to silencing via RNA interference (Volpe *et al.*, 2002, 2003). Beyond chromosomal integrity, there are hints that RNA

contributes to macroscopic cellular architecture. In *Xenopus* oocytes, a noncoding RNA called Xlsirts integrates directly into the cytoskeletal network as an essential structural element, among other functions (Kloc *et al.*, 2005, 2007). Additional work in *Xenopus* extracts has shown that bulk RNAs associate directly with the mitotic spindle and are necessary for spindle assembly independent of any translational activity (essentially, functioning as ncRNAs; Blower *et al.*, 2005).

Other ncRNAs act as sequence-specific guides for assorted maintenance tasks. Small nucleolar RNAs (snoRNAs) position the site-specific chemical modifications of their noncoding relatives (rRNA, tRNA, snRNA) (Ganot *et al.*, 1997, 1999; Jady and Kiss, 2001; Kiss-Laszlo *et al.*, 1996; Ni *et al.*, 1997; Omer *et al.*, 2000; Tycowski *et al.*, 1998). These small RNAs can also perform the odd side job, as in the case of one recently described snoRNA that biases exon choice during splicing of a complementary mRNA (Kishore and Stamm, 2006). Kinetoplastids such as trypanosomes also take advantage of the sequence specificity of small guide RNAs to direct the massive reshaping of their mitochondrial RNA repertoire through editing (specifically, uracil insertions and deletions) (Benne *et al.*, 1986; Feagin *et al.*, 1987).

2.2. Small ncRNAs and posttranscriptional gene silencing

This capacity for sequence-specific guidance is a key attribute of RNA-mediated gene regulation. In the growing catalog of functional ncRNAs, the smallest versions feature prominently in posttranscriptional gene repression. Both exogenous and endogenous sources of double-stranded RNA give rise to 21–25 nucleotide small interfering RNAs (siRNAs) (Fire *et al.*, 1998; Hamilton and Baulcombe, 1999; Hammond *et al.*, 2001; Liu *et al.*, 2004) and microRNAs (miRNAs), respectively (Bartel, 2004; Lee *et al.*, 1993; Wightman *et al.*, 1993). These tiny noncoding chains are sufficient to guide silencing complexes to complementary mRNA targets, leading to mRNA cleavage or destabilization (in the case of siRNAs and miRNAs) or translational repression (in the case of miRNAs) (Bartel, 2004; Mansfield *et al.*, 2004; Yekta *et al.*, 2004). Metazoan germ cells express a wholly distinct class of 25–31 nucleotide piRNAs (Piwi-interacting small RNAs) that silence transposons via DNA methylation (Aravin *et al.*, 2008; Hartig *et al.*, 2007; O'Donnell and Boeke, 2007). Despite the advent of highly sophisticated protein-based mechanisms of gene regulation, small ncRNA-mediated pathways have persisted throughout evolution (Grimson *et al.*, 2008), shaping gene expression profiles and reinforcing genomic integrity along the way.

Since their discovery, these small RNAs, particularly the miRNAs, have been subject to intense investigation (for review, see Bartel 2004, 2009). An estimated 30–60% of eukaryotic genes are subject to miRNA

regulation (Friedman *et al.*, 2009; Lewis *et al.*, 2003; Yu *et al.*, 2007), implicating this mechanism as a substantial means by which organisms modulate their gene expression profiles. Unsurprisingly, miRNA-mediated regulation pervades through diverse aspects of immune cell development and function. As many of these findings have been recently reviewed (Lu and Liston, 2009; Petrocca and Lieberman, 2009; Xiao and Rajewsky, 2009), we will instead focus our attention on the longer relatives of these regulatory ncRNAs.

2.3. Transcription-associated ncRNAs

Recent examinations of transcriptional landscapes have revealed an astounding expanse of transcriptional activity throughout mammalian genomes, far exceeding the number of protein-coding genes (Birney *et al.*, 2007; Kapranov *et al.*, 2007b; Okazaki *et al.*, 2002). These transcriptome maps depict an interlaced system of coding and noncoding units, yielding a considerable population of unannotated, uncharacterized long ncRNAs. Though some of these noncoding transcripts may indeed represent nonspecific transcriptional noise (Struhl, 2007), recent studies indicate that both the process and the product of noncoding transcription are likely to be genuinely functional.

Bidirectional promoter activity contributes substantially to this pervasive transcription. In yeast, the long noncoding products of this phenomenon have been named cryptic unstable transcripts (CUTs) and stable unannotated transcripts (SUTs) (Neil *et al.*, 2009; Wyers *et al.*, 2005; Xu *et al.*, 2009). These ncRNAs emanate from nucleosome-free domains at promoters and 3′ termini of protein-coding units. Promoter-associated CUTs and SUTs arise bidirectionally, giving rise to noncoding transcripts that can overlap with neighboring mRNAs in either sense or antisense orientations. A few case studies have shown that individual CUTs can mediate transcriptional silencing of proximal genes, though the molecular mechanism is unclear (Berretta *et al.*, 2008; Bird *et al.*, 2006; Camblong *et al.*, 2007; Hongay *et al.*, 2006). The ubiquity and genic association of these CUTs and SUTs in the yeast transcriptome hint at a fundamental mechanism of regulating gene expression.

Mammalian transcriptome profiles follow a strikingly similar pattern, with clusters of ncRNAs arising at boundaries of transcribed genes. Long, unstable promoter upstream transcripts (PROMPTs) initiate bidirectionally ~0.5–2.5 kb upstream of transcription start sites (Preker *et al.*, 2008). The genomic addresses of PROMPTs are enriched in markers of active transcription, such as RNA polymerase II (Pol II) and acetylated histone H3 lysine 9, but do not associate with transcription initiation factors that mark coding loci (Preker *et al.*, 2008). PROMPTs partially overlap with distinct class of bidirectional promoter-associated long RNAs (PALRs) (Kapranov *et al.*, 2007a). These PALRs initiate proximal to transcription

start sites, with the resulting ncRNA often overlapping the first exon and intron of the neighboring coding unit. Several investigators have also independently identified short (<200 nucleotides) noncoding transcripts (of varying size and stability) associated with the 5′ and 3′ termini of genes (Core *et al.*, 2008; Kapranov *et al.*, 2007a; Seila *et al.*, 2008). The promoter-associated subset of these short ncRNAs overlaps with PALRs (though not PROMPTs), suggesting that they may represent processed versions of long ncRNAs associated with active transcription. These recent discoveries depict a highly active transcriptional landscape, where induction of discrete protein-coding genes is accompanied by a flurry of proximal noncoding transcription. This system of pervasive genic and intergenic transcriptional activity, inherently bidirectional promoters, and widespread polymerase pausing at promoters is not fully understood, but has been proposed to alter DNA accessibility, to create negative supercoiling to promote transcription initiation, or to poise pools of Pol II molecules for rapid activation of associated genes.

2.4. Long ncRNAs

Recent bioinformatic searches for conserved long ncRNAs indicate that they comprise a small but substantial pool of *bona fide* functional species that are likely to regulate many processes (Guttman *et al.*, 2009). Only a few orphan examples of such regulatory long ncRNAs have been characterized—but from these few case studies, a startling diversity of regulatory modes has been uncovered.

Several of these regulatory long ncRNAs can be broadly categorized as modulators of DNA accessibility, several examples of which have been documented in yeast. Low-abundance noncoding transcription through promoters of coding genes can remodel the chromatin configuration to favor RNA polymerase access. This read-through may arise in the same orientation as the coding gene (e.g., the fission yeast *fbp1* locus) or in an antisense orientation (e.g., the budding yeast *pho5* locus) (Hirota *et al.*, 2008; Uhler *et al.*, 2007). The cascade of chromatin disruption traveling toward the promoter appears to facilitate polymerase engagement of the coding unit. A similar case of intergenic transcription is proposed to regulate chromatin opening at the human *β-globin* locus (Gribnau *et al.*, 2000).

In contrast, other ncRNAs associated with coding loci promote transcriptional repression through a variety of mechanisms. This may take the form of transcriptional interference *in cis* at promoter regions (Osato *et al.*, 2007). One instance of this takes place at the *SER3* gene in budding yeast. Noncoding transcription through the upstream regulatory region of this

gene (producing a ncRNA called Srg1) inhibits the binding of transcriptional activators to the promoter, effectively repressing *SER3* expression (Martens *et al.*, 2004). Cryptic transcription can also induce repressive histone modifications in a proximal coding locus, as in the case of the yeast *GAL1-10* gene cluster (Houseley *et al.*, 2008; Pinskaya *et al.*, 2009). A noncoding transcript initiating upstream of the human dihydrofolate reductase promoter mediates transcriptional repression by a unique mechanism. This ncRNA forms a stable triplex with the promoter DNA and also interacts directly with the general transcription factor TFIIB to favor disassociation of the preinitiation complex from the promoter (Martianov *et al.*, 2007). Interestingly, in this particular case, RNA-dependent repression was also observed when the ncRNA was experimentally supplied *in trans*, indicating that the RNA itself, and not simply transcription, was functionally important.

Incidentally, short ncRNA inhibitors of the core transcriptional machinery exist throughout phylogeny: the bacterial 6S RNA, the murine B2 RNA, and human SINE-derived Alu RNAs (Espinoza *et al.*, 2004; Kettenberger *et al.*, 2006; Mariner *et al.*, 2008; Wassarman and Storz, 2000). These RNAs bind directly to the polymerase, in some cases competing with promoter DNA for access to the active site. In addition to ncRNA inhibitors of transcriptional initiation, vertebrates also possess an RNA-based system to negate transcriptional elongation. The elongation factor P-TEFb phosphorylates Pol II to generate an elongating transcriptional complex. Elongation is obstructed, however, when the noncoding transcript 7SK binds and represses the kinase activity of P-TEFb (Nguyen *et al.*, 2001; Yang *et al.*, 2001).

Long ncRNAs also control gene expression through secondary interactions with transcriptional cofactors. The murine developmental program illustrates one such mechanism of transcriptional regulation *in cis*. The *Dlx5/6* homeodomain gene cluster (involved in limb patterning and neuronal development) produces an intergenic ncRNA, Evf-2, which binds another homeodomain protein (Dlx2) to cooperatively activate *Dlx5/6* enhancer activity (Feng *et al.*, 2006). ncRNA-mediated transcriptional repression *in cis* has also been observed in the cellular response to genomic insults. General DNA damage signals induce noncoding transcription upstream of the mammalian *CCND1* locus, which encodes a cell cycle regulator that is repressed upon DNA damage. This site-specific ncRNA tether recruits the RNA-binding protein TLS, which then inhibits histone acetyltransferase activity at the downstream *CCND1* gene (Wang *et al.*, 2008).

Noncoding RNAs need not arise from the same genomic location as their regulated targets. For example, the vertebrate heat-shock response exhibits one mode of RNA-induced transcriptional activation. A long ncRNA, called HSR1, induces the trimerization of the heat-shock

transcription factor HSF1, activating its capacity to stimulate expression of downstream targets (Shamovsky *et al.*, 2006). It is thought that this constitutively expressed ncRNA undergoes temperature-dependent conformational changes that allow it to mediate HSR1 oligomerization. *Trans*-acting ncRNAs can also function as potent transcriptional repressors. The human *Hox* genes, which are responsible for developmental body patterning, cluster in several discrete genomic loci. A recently discovered intergenic ncRNA, HOTAIR, originates from the *HoxC* cluster, but targets a distal *HoxD* gene cluster for Polycomb-mediated epigenetic silencing (Rinn *et al.*, 2007). The influence of ncRNA also extends beyond transcriptional processes *per se*. The subcellular trafficking of the transcription factor NFAT (nuclear factor of activated T cells) serves as a prime example. The noncoding NRON (noncoding repressor of NFAT) RNA interacts with nuclear importins and somehow obstructs the ability of NFAT to access its transcriptional targets (Willingham *et al.*, 2005). These examples of protein-associated regulatory ncRNAs hint at a vast capacity for cooperation between RNA- and protein-based mechanisms of gene regulation.

The prevalence of sense and antisense transcript pairs suggests that many opportunities for RNA duplexing may arise in mammalian transcriptomes (Katayama *et al.*, 2005; Okazaki *et al.*, 2002). In budding yeast, one of the exceptional organisms lacking RNAi machinery, endogenous long antisense RNAs can silence complementary sense mRNAs (Camblong *et al.*, 2009). It remains to be seen whether similar RNAi-independent pathways exist in higher organisms. Overlaps between sense mRNA and antisense ncRNA pairs can also mask regulatory motifs in the coding mRNA. Antisense ncRNAs intersecting with mRNA splice sites can influence the production of full-length mRNAs or exon choice during alternative splicing (Beltran *et al.*, 2008; Hastings *et al.*, 2000; Lazar *et al.*, 1990; Rintala-Maki and Sutherland, 2009). Other instances of sense–antisense RNA pairs have been proposed to regulate RNA editing and localization (Kumar and Carmichael, 1997), RNA stability (via masking of AU-rich sites in mRNA 3′-UTRs) (Capaccioli *et al.*, 1996), and even translation (Li and Murphy, 2000). In addition to the potential for regulation via sense–antisense RNA base pairing, one must also consider the mechanics of bidirectional transcription across a single locus. Collisions between converging polymerases may create transcriptional pauses that can quantitatively affect gene expression or stabilize melted DNA structures that are accessible to other regulatory factors. The negative and positive DNA supercoils that trail and lead an elongating polymerase, respectively, may be amplified in the case of bidirectional transcription. These exceptional stresses placed on the local chromatin environment may create additional opportunities for DNA-topology-dependent gene regulation.

Some of the best characterized long ncRNAs have been implicated in epigenetic programming and imprinting. Long ncRNAs nucleate two distinct mechanisms of dosage compensation (adjustment of the male XY versus female XX chromosome inequity). X chromosome inactivation in female cells of placental mammals stems from the mutually exclusive expression of two ncRNAs: Xist (expressed from the inactive X chromosome) and its antisense counterpart Tsix (expressed from the active X chromosome). Xist coating of the inactive X chromosome promotes heterochromatic silencing through repressive histone modifications and DNA methylation (Borsani *et al.*, 1991; Brown *et al.*, 1991; Panning and Jaenisch, 1996; Panning *et al.*, 1997). In contrast, dosage compensation in *Drosophila* follows the opposite route, where the single male X chromosome undergoes hypertranscription, mediated by a RNP complex containing the ncRNAs roX1 and roX2 (Ilik and Akhtar, 2009). A handful of imprinted gene clusters are also associated with ncRNAs: H19 (Bartolomei *et al.*, 1991), Nespas (Wroe *et al.*, 2000), Air (Sleutels *et al.*, 2002), Kcnq1ot1 (Pandey *et al.*, 2008)—which are believed to participate in the epigenetic silencing of their respective loci.

Long ncRNAs represent an expansive class of regulatory molecules that touch on multiple aspects of gene expression. Here, we have discussed only a few functional examples that speckle the regulatory landscape. Given the ubiquity of these RNAs in the transcriptome, there are undoubtedly many more examples and mechanisms of ncRNA function to be uncovered. It remains to be seen how (and if) these characterized ncRNAs relate to the extensive populations of yeast CUTs/SUTs or the mammalian PROMPTs and PALRs and if there are indeed broad classes of ncRNAs that act as fundamental genomic regulators.

3. ncRNAs AND THE ADAPTIVE IMMUNE SYSTEM

The mammalian immune system provides an excellent setting for asking just these types of questions, either from the perspective of broad gene regulation by ncRNAs or from the perspective of individual ncRNA involvement in specific immunological phenomena. Systematic identification of long ncRNAs in discrete developmental and functional immune cell subsets is highly feasible, given our ability to purify or sort out these subsets by surface markers. These approaches are already underway, as demonstrated in a recent publication cataloging long ncRNAs in mammalian $CD8^+$ T lymphocytes (Pang *et al.*, 2009). Furthermore, idiosyncratic processes in certain immune cell types have long been associated with noncoding transcripts of unknown function. We allude specifically to the sterile (or germline) transcripts that accompany somatic rearrangements

of antigen receptor genes in mammalian lymphocytes, to be discussed in more detail below.

3.1. Noncoding transcription in V(D)J recombination

The immune system serves as a striking example where the *de facto* information content of the genome is not sufficient for full biological function. Vertebrates encounter innumerable pathogenic and environmental insults throughout their lifetimes, and thus require a recognition system capable of identifying these infinitely diverse particles as nonself. This demand for diversity in recognition certainly exceeds the amount of information that can be encoded in the entire genome, much less in one specific locus.

B and T lymphocytes of the adaptive immune response take on this dilemma as they synthesize the cell surface receptors responsible for recognizing antigens—immunoglobulins (Ig) and T cell receptors (TCR), respectively. To achieve the requisite recognition and functional diversity, these antigen receptor genes undergo somatic alterations during lymphocyte development. Ig and TCR diversification follow roughly the same template, but here we will focus mainly on B lymphocytes and Ig diversification.

The first step of Ig gene diversification, V(D)J recombination, occurs in pro-B cells as they mature in the bone marrow. This process involves the ordered joining of three gene modules—variable (V), diversity (D), and joining (J)—to reconstitute a functional V region (Early *et al.*, 1979, 1980; Maki *et al.*, 1980; Sakano *et al.*, 1981) (Fig. 2.1A). Recombination signal sequence motifs flanking V, D, and J segments are recognized and cleaved by the molecular engines of the V(D)J recombinase—the RAG1 and RAG2 proteins (McBlane *et al.*, 1995; Schatz and Baltimore, 1988; Schatz *et al.*, 1989; van Gent *et al.*, 1995). Nonhomologous end-joining machinery subsequently ligates the appropriate DNA ends (Gao *et al.*, 1998; Grawunder *et al.*, 1998; Li *et al.*, 1995; Moshous *et al.*, 2001; Taccioli *et al.*, 1993). Each mature B lymphocyte that emerges from the bone marrow after V(D)J recombination is then distinct from all others at three levels: (1) the random choice of V, D, and J from an extensive pool of germline gene segments, (2) the combination of rearranged heavy and light chains, and (3) junctional insertions and deletions which occur during rearrangement. Together, this combinatorial and junctional diversity can produce a repertoire of distinct Ig variable region specificities in excess of 10^7.

Regulation of locus accessibility has been thought to provide the temporal control over this lineage- and stage-specific reaction (Schlissel and Stanhope-Baker, 1997; Stanhope-Baker *et al.*, 1996). At the Ig heavy chain locus, targeted histone modifications that are characteristic of active loci foreshadow the ordered recombination of D to J, followed by V to DJ

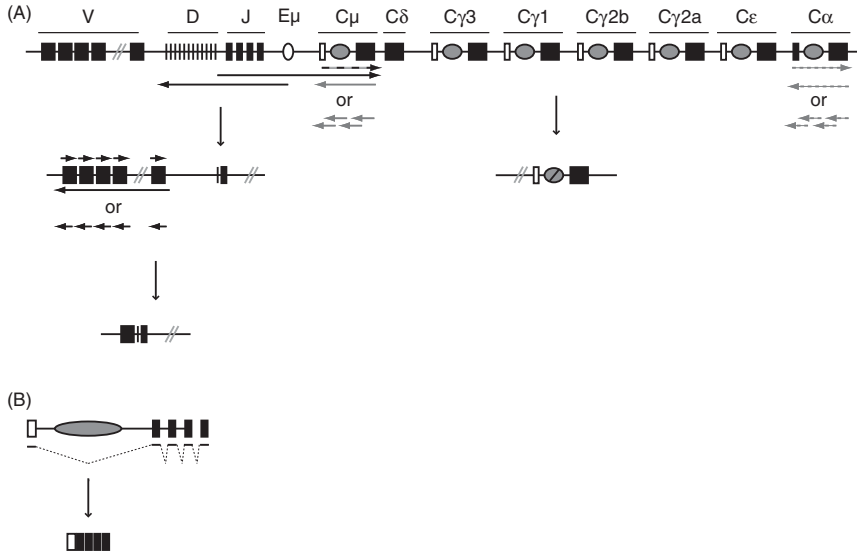

FIGURE 2.1 Noncoding transcription across antigen receptor loci. (A) The mouse Ig heavy chain locus is diagrammed (not to scale). Black boxes denote exon segments (with respect to V, D, and J segments) or sets of constant region exons, the white circle denotes the intronic Eμ enhancer, white boxes denote the germline I exons, and gray ovals denote switch (S) regions. The upstream variable region exon is assembled from V, D, and J gene segments, where D–J joining precedes V–DJ joining. The locations of noncoding transcripts are shown in black, where arrows pointing to the right indicate sense transcription, and arrows pointing to the left indicate antisense transcription. At the downstream constant region, class switching from IgM (Cμ) to IgA (Cα) is shown, with accompanying sense and antisense noncoding transcription represented by gray arrows. The dashed arrows at the Cα indicate inducible transcription (as opposed to constitutive transcription at Cμ). (B) The sense germline transcript from the Cμ locus is depicted (again, not to scale). Locus elements are color coded as in (A). The nascent RNA is spliced to exclude the S region and C region introns. This yields a mature germline transcript consisting of the I exon attached to the C region exons.

(Chowdhury and Sen, 2001; Johnson *et al.*, 2003, 2004; Maes *et al.*, 2001; Su *et al.*, 2003). How are these ordered chromatin modifications established? One correlative explanation springs from the noncoding transcription that synchronizes with Ig locus reorganization.

Before D to J rearrangement, two distinct "germline" transcripts (referring to the locus in its unrearranged, or germline state) arise either from the 3′-most D_H promoter or from the intronic enhancer (Eμ) and extend downstream through the first heavy chain constant region gene (Lennon and Perry, 1985; Reth and Alt, 1984). Later, as V to DJ rearrangement begins, the V region segments undergo extensive germline transcription, though this process does not correlate particularly well with V

region choice during recombination (Angelin-Duclos and Calame, 1998; Yancopoulos and Alt, 1985). Though the production of these ncRNAs correlates suspiciously well with the timing and location of rearranging gene segments, their exact role has not been determined. It is possible that germline transcription disrupts chromatin in a stepwise manner to nucleate locus opening of the appropriate V, D, or J gene clusters. In parallel, the Ig heavy chain locus also undergoes noncoding transcription in the opposite orientation. Prior to D–J recombination, antisense transcription initiates from the intronic Eμ enhancer and traverses the entire D_H cluster (Bolland *et al.*, 2007). During or after D–J recombination, a second wave of antisense transcription navigates through the V region cluster, seemingly in preparation for subsequent V–DJ recombination (Bolland *et al.*, 2004). The mechanical opening of the appropriate gene clusters is perhaps accompanied by cotranscriptional recruitment of chromatin remodeling factors that trail along with an elongating polymerase (Belotserkovskaya *et al.*, 2003; Cho *et al.*, 1998; Wittschieben *et al.*, 1999). In addition to establishing local chromatin opening, active transcription is also known to promote higher order chromatin decondensation (Muller *et al.*, 2001). In their germline configuration, the V, D, and J segments span rather large genic and intergenic distances. Some degree of DNA flexibility must be attained in order for the appropriate gene segments to align in the correct configuration for recombination (Jhunjhunwala *et al.*, 2009). Transcription-dependent unfolding of the chromatin fiber, particularly if it occurs in a stepwise manner, could be extremely useful in facilitating the timed three-dimensional repositioning of DNA bits that will eventually participate in V(D)J recombination.

The latest evidence for functional ncRNAs during antigen receptor assembly comes from T lymphocytes undergoing TCRα recombination. This locus, a variation on the general theme of antigen receptor organization, contains a cluster of V segments, followed by an uncommonly large cluster of J segments. Noncoding transcription through the J array preferentially activates the 5′ J segments for V–J recombination, while suppressing usage of 3′ J segments through transcriptional interference (Abarrategui and Krangel, 2006, 2007). Furthermore, the 5′-most J segments are enriched in methylated histone H3 lysine 4 in a transcription-dependent manner, thus specifying a preferred binding domain for Rag2 (Matthews *et al.*, 2007). The causative links between noncoding transcription (or noncoding transcripts themselves) and histone modifications have yet to be described, however.

3.2. ncRNAs and class switch recombination

Mature B lymphocytes that have successfully undergone V(D)J recombination exit the bone marrow and circulate through the bloodstream to the

peripheral lymphoid organs, where they undergo secondary Ig diversification. Activation-induced cytidine deaminase (AID) converts cytidine to uracil at discrete spots in the Ig locus to initiate two distinct diversification reactions (Arakawa *et al.*, 2002; Muramatsu *et al.*, 1999, 2000). AID-induced lesions in the assembled Ig variable region nucleate somatic hypermutation of the locus, creating variants with increased affinity for a cognate antigen. In contrast, deamination of cytidines in the Ig heavy chain constant regions results in DNA rearrangement. This process of class switch recombination (CSR) swaps the initially expressed Cμ constant region for an alternate downstream constant region to change the effector function associated with a particular Ig (Fig. 2.1A). A repetitive G/C-rich DNA element called the switch (S) region precedes each set of alternative C region exons (with the exception of Cδ). The S regions serve as the substrates for AID-mediated deamination, as well as the sites for nonhomologous recombination. The two processes of somatic hypermutation and CSR cooperate to generate Igs that are optimized for each immune response.

AID activity hinges on transcription of target DNA, and thus both somatic hypermutation and CSR track very closely with transcription signatures across the Ig locus (Bachl *et al.*, 2001; Fukita *et al.*, 1998; Lee *et al.*, 2001; Peters and Storb, 1996). At the constant regions, however, transcription is required in a noncoding capacity. As the unrearranged constant region genes become activated for DNA recombination, they undergo germline transcription. The sterile read-through initiates from the Ig heavy chain enhancer (Eμ) for the Cμ region or from independent promoters located in each of the downstream C regions and then traverses a noncoding exon (designated "I"), the S region, and the C region exons (Berton *et al.*, 1989; Esser and Radbruch, 1989; Gerondakis, 1990; Lebman *et al.*, 1990; Lutzker *et al.*, 1988; Rothman *et al.*, 1990b; Stavnezer-Nordgren and Sirlin, 1986; Xu and Stavnezer, 1990). The transcript subsequently undergoes splicing to remove the intronic S region and the C region introns (Fig. 2.1B). Site-specific germline transcription invariably precedes the induction of CSR to a particular constant region (Gerondakis, 1990; Lebman *et al.*, 1990; Lutzker and Alt, 1988; Lutzker *et al.*, 1988; Radcliffe *et al.*, 1990; Rothman *et al.*, 1990a,b; Stavnezer *et al.*, 1988; Xu and Stavnezer, 1990). Stop codons litter all three reading frames of the I exons, so the transcripts are unlikely to retain any protein-coding capacity. Furthermore, B lymphocytes harboring a heterologous sequence in place of the I exon can still potentiate CSR (Harriman *et al.*, 1996; Qiu *et al.*, 1999). However, mutation or deletion of the germline promoters leads to CSR defects, indicating that either the process of transcription or the ncRNA itself is functionally important (Bottaro *et al.*, 1998; Jung *et al.*, 1993; Zhang *et al.*, 1993).

The noncoding transcription through constant regions is one of the earliest locus-specific events preceding CSR and is necessary for

recombination (Wakatsuki and Strober, 1993; Xu *et al.*, 1993; Zhang *et al.*, 1993). As for germline transcripts associated with V(D)J recombination, transcriptional activity has been proposed to regulate constant region accessibility or targeting. Localized transcriptional activity would certainly promote the formation of an open chromatin domain, and, accordingly, active histone marks at S regions do correlate with germline transcription (Nambu *et al.*, 2003; Wang *et al.*, 2009). However, no direct causal relationship has been established between noncoding transcription and chromatin remodeling of the S regions. More interestingly, AID is coordinately recruited to S regions with Pol II, suggesting an opportune means of AID localization. Preparatory noncoding transcription could poise AID molecules at the S regions that will eventually undergo deamination, synapsis, and recombination. This also has implications for intralocus targeting of AID activity, as somatic hypermutation and CSR are thought to occur exclusively of one another.

Another functional contribution of germline transcripts relates to creation of accessible single-stranded DNA substrates for AID, whether in the context of a transcriptional bubble or in the context of an R-loop (G-rich RNA hybridized to its cognate DNA). Generation of these R-loops would expose an unpaired DNA strand for AID-mediated deamination. The G/C-rich S regions are supremely amenable to the formation of such structures, and, indeed, there is evidence of R-loop formation in mammalian S regions (Daniels and Lieber, 1995; Huang *et al.*, 2006; Mizuta *et al.*, 2003; Reaban and Griffin, 1990; Reaban *et al.*, 1994; Yu *et al.*, 2003). In further support of this model, S region function depends highly on the orientation in which it is transcribed, where only transcription in the physiological orientation (which favors R-loop formation) supports efficient CSR (Shinkura *et al.*, 2003; Tian and Alt, 2000). *In vitro* studies suggest that these R-loops form in the wake of RNA polymerase, where the nascent RNA competes with the nontemplate DNA strand for annealing to the template DNA strand (Roy and Lieber, 2009; Roy *et al.*, 2008). Alternatively, R-loops could arise from stable extended transcription bubbles that have failed to denature after transcription. The *in vivo* mechanism of R-loop formation at S regions has yet to be determined.

High densities of elongating RNA polymerases have been detected at S regions (Wang *et al.*, 2009), possibly reflecting extensive pausing of polymerases at the locus. We imagine several distinct (though perhaps overlapping) roles for paused germline transcription in CSR. These stalling events could arise as successive polymerases traveling across a single S region collide with thermodynamically stable R-loops that have been established by a preceding transcriptional event. Simultaneously, the stalled polymerases could deliver bound AID molecules to this accumulated reservoir of "frozen" single-stranded DNA substrates. Alternatively,

physical obstacles to polymerase elongation (such as stable R-loops) may stimulate cycles of polymerase disengagement and reloading, thus ensuring continued delivery of AID to the S regions. Finally, stalled polymerase complexes have been found to act as potent local insulators (Chopra *et al.*, 2009), restricting the recruitment of enhancers to proximal genes. Paused germline transcription may therefore be important in directing the specific recruitment of Ig heavy chain enhancer elements to the Ig constant region and not to the upstream Ig variable region (Wuerffel *et al.*, 2007), while also favoring the deposition of AID at S regions but not the upstream variable region. This would serve as an effective means of functionally separating somatic hypermutation and CSR.

The R-loop model for germline transcript function is probably the most extensively tested hypothesis to date, but many open questions remain. The G/C-poor S regions in amphibians are still capable of mediating CSR, suggesting that R-loop-dependent mechanisms of regulating substrate accessibility may have manifested recently in evolution. Thus, the magnitude of R-loop contribution to CSR is uncertain. It is also unclear how an *in vivo* R-loop at the S region would be resolved and if this model would be consistent with the kinetics of germline transcript splicing (since the transcribed S region is excised as an intron and may exist only transiently). In addition, the C-rich strand of the S region, incapable of forming an R-loop, would require a distinct mechanism to generate single strandedness.

One puzzling piece of evidence suggests a more complex role for germline transcripts in regulating CSR. *In vivo* experiments have previously demonstrated that genetic ablation of I exon splice donor sites severely hinders CSR (Hein *et al.*, 1998; Lorenz *et al.*, 1995). It remains a mystery how splicing of a wholly noncoding RNA could affect a downstream DNA recombination event. The cotranscriptional processing of nascent germline transcripts—capping, splicing, and polyadenylation—may function outside of direct RNA maturation and instead allow for secondary recruitment of downstream factors. For example, the RNA polyadenylation complex has recently been shown to interact with DNA repair factors such as Ku70 and DNA-PK, both of which are involved in end-joining during CSR (Shi *et al.*, 2009). One could imagine a similar role for the spliceosome, an enormous complex that includes many protein subunits not directly involved in RNA splicing (Zhou *et al.*, 2002). Perhaps splicing of the germline transcripts is not necessary *per se*, but rather preassembles downstream effectors at the Ig heavy chain locus. Strikingly, a spliceosome-associated protein called CTNNBL1 interacts with AID, and this interaction contributes to AID-dependent diversification events (Conticello *et al.*, 2008). The intronic by-products of splicing could also be functionally important. Occasional examples of highly stable introns have been reported (Clement *et al.*, 1999, 2001). Splicing of

full-length germline transcripts could serve to generate local concentrations of short, stable S region introns that can support R-loop formation.

Mirroring the bidirectional transcription that accompanies rearrangement of the Ig variable region, antisense transcription through the constant region also precedes CSR. These low-abundance transcripts initiate from heterogeneous start sites throughout the constant region and are produced in parallel with their sense counterparts (constitutively from the Cμ locus and inducibly at downstream C loci) (Chowdhury *et al.*, 2008; Perlot *et al.*, 2008). The 5′-end heterogeneity and low abundance of antisense transcripts may indicate passive, promoter-independent polymerase activity. Antisense promoters have not been identified in the mouse Ig constant region, though two have been identified in Sμ of a human Burkitt's lymphoma cell line (Apel *et al.*, 1992). Baseline bidirectional activity of the germline I promoters could be involved, though this is unlikely, as each germline promoter responds to different activating stimuli. The finer details of these antisense RNAs must be investigated—whether they represent single long RNAs or multiple abortive transcripts across each constant region, whether they undergo standard RNA processing, and whether they regulate their sense counterparts through base pairing. In any case, bidirectional transcription seems to be a convenient mechanism to promote polymerase stalling at S regions. Collided transcriptional complexes could further stabilize R-loops generated from sense germline transcription, while freezing the transcriptional bubble in the opposite orientation to allow AID access to both strands.

Germline transcription at the Ig constant regions marks a fascinating convergence of ncRNAs, bidirectional transcription, RNA processing, and DNA repair. The vast (and growing) functional space of ncRNAs indicates that these germline transcripts may well account for some of the unexplained features of antigen receptor rearrangement, particularly locus accessibility and targeting. Since the function of these noncoding transcripts is only vaguely understood, they present an exciting opportunity to explore ncRNA-mediated gene regulation. Given the prevalent usage of ncRNAs as guides, it is tempting to speculate that germline transcripts may play an additional part in targeting effector proteins to the constant regions. Whether germline transcripts harbor primary sequence motifs or secondary structures that attract protein partners remains to be shown. Is it also possible that noncoding transcription modulates chromatin topology of the Ig constant regions beyond simple transcriptional opening? Long-range interactions between Ig heavy chain enhancer elements and germline promoters have been observed in B lymphocytes undergoing CSR (Wuerffel *et al.*, 2007). This may reflect a linked system of transcription and chromatin reorganization that extrudes the Ig heavy chain DNA from compacted chromosome territories, allowing the locus the flexibility to position the necessary regions in close proximity.

Extending our perspective beyond lymphocyte biology, meiotic recombination hotspots have been found to correlate with sites of noncoding transcription (Nishant *et al.*, 2004; Wahls *et al.*, 2008). Perhaps germline transcription and CSR represent a general paradigm for DNA recombination (and illegitimate translocations, when gone awry).

4. PERSPECTIVE

In recent years, extensive noncoding transcription has been observed on a genome-wide scale, with signs of evolutionary conservation (Guttman *et al.*, 2009). Computationally, these sites tend to cluster with genes involved in transcriptional regulation and are unlikely to represent transcriptional noise. Widespread bidirectional transcription has also been identified, representing at least two distinct phenomena. The mammalian transcriptome includes copious numbers of coordinated sense and antisense transcript pairs (Chen *et al.*, 2004; Katayama *et al.*, 2005; Kiyosawa *et al.*, 2003; Yelin *et al.*, 2003), where antisense interference may generally regulate abundance of the sense strand. More recently, a distinct phenomenon of diverging bidirectional transcription at promoters of active genes has been observed (Core *et al.*, 2008; He *et al.*, 2008; Preker *et al.*, 2008; Seila *et al.*, 2008; Xu *et al.*, 2009) and is proposed to influence promoter regulation and accessibility. Germline transcription of antigen receptor loci appears to incorporate aspects from each of these processes, which may inform our conception of how ncRNA contributes to antigen receptor diversification. We expect that global transcriptome profiling in lymphocytes will reveal the true extent of interleaved transcripts across these sites and also provide starting points for understanding other aspects of immune function and diseases of immune etiology.

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