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Review

Architectural and Functional Commonalities between Enhancers and Promoters

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With the explosion of genome-wide studies of regulated transcription, it has become clear that traditional definitions of enhancers and promoters need to be revisited. These control elements can now be characterized in terms of their local and regional architecture, their regulatory components, including histone modifications and associated binding factors, and their functional contribution to transcription. This Review discusses unifying themes between promoters and enhancers in transcriptional regulatory mechanisms.

Recent genome-wide studies have significantly advanced our understanding of the genomic architecture that underlies gene expression in higher eukaryotes. Integrative analyses of the transcriptome, transcription factor (TF) binding profiles, and epigenomes reveal complex organization of individual transcription units scattered throughout the genome and causal relationships among the regulatory DNA sequences, chromatin state, and transcriptional activity. In particular, a considerable amount of data have established that enhancers are not merely a collection of TF binding sites, but also have the capacity to drive transcription independent of their target promoters. This feature of enhancers suggests that they serve more regulatory functions than previously appreciated.

Regulatory DNA Elements in Eukaryotic Gene Expression

Transcription of a gene in eukaryotes is a highly complex process that requires precise coordination in the assembly of *trans*-acting factors through the recognition of various types of regulatory DNA sequences. The promoter and the enhancer represent DNA regulatory regions responsible for ensuring proper spatiotemporal expression patterns of eukaryotic genes. The promoter generally refers to a DNA region that allows accurate transcription initiation of a gene (Smale and Kadonaga, 2003). The core promoter is a minimal stretch of DNA sequences (e.g., the TATA box, initiator, and downstream core promoter element) surrounding the transcription start site that directly interacts with the components of basal transcription machinery, including RNA polymerase II (RNAPII). Although the DNA sequences or motifs comprising the core promoter region for individual genes can be structurally and functionally diverse, its universal role is thought to drive accurate transcription initiation (Smale and Kadonaga, 2003). Transcription factors that bind ~100–200 bp upstream of the core promoter can increase the rate of transcription by facilitating the recruitment or assembly of the basal transcription machinery onto the core promoter or by mediating the recruitment of specific distal regulatory DNA sequences to the core promoter (Akbari et al., 2008).

These distal sequences, known as enhancers, activate or increase the rate of transcription from the target gene promoter independent of their position and orientation with respect to target genes (Maniatis et al., 1987). In multicellular organisms, enhancers are primarily responsible for the precise control of spatiotemporal patterns of gene expression. Enhancer elements were initially discovered in the early 1980s in studies that characterized eukaryotic gene promoters. Functional tests of sea urchin histone gene expression in the *Xenopus oocyte* identified DNA sequences located upstream of the TATA box motif that positively influence H2A gene transcription, originally termed transcriptional ''modulators'' (Grosschedl and Birnstiel, 1980). Deletion of the modulator resulted in 15- to 20-fold decrease in H2A gene expression. Interestingly, the modulator activity was retained even when its DNA sequence was inverted. Similarly, the tandem 72 bp DNA repeats located upstream of viral SV40 early gene were found to be indispensable for SV40 early gene expression (Benoist and Chambon, 1981). Shortly after those initial observations, a series of studies on the SV40 enhancer established the conceptual framework for defining enhancers as follows (Atchison, 1988; Banerji et al., 1981; Fromm and Berg, 1982, 1983; Khoury and Gruss, 1983; Moreau et al., 1981): (1) Enhancers increase transcription of a linked gene from its correct initiation site specified by the core promoter, (2) enhancer activity is independent of orientation relative to its target gene, (3) enhancers can function independent of their position relative to the target genes, and also over long distances, (4) enhancers can function with a heterologous promoter, (5) enhancers exhibit DNase I hypersensitivity (HS), which reflects a less compacted chromatin state as a result of the binding of various transcription factors. Although these properties were defined more than three decades ago, they are still widely used to classify enhancers.

Subsequent studies identified the first mammalian cellular enhancer, which is required for efficient expression of the immunoglobulin (*Ig*) heavy-chain gene (Banerji et al., 1983; Gillies

et al., 1983; Neuberger, 1983). Importantly, the *Ig* enhancer studies provided the first evidence demonstrating that enhancer activity exhibits tissue or cell-type specificity. When various cell lines were tested, *Ig* enhancer activity was observed only in lymphocyte-derived cell lines (Banerji et al., 1983; Gillies et al., 1983). Since then, a variety of cell-type- or developmental stage-specific enhancers have been determined to regulate the expression of genes in higher organisms (Müller et al., 1988). Transcriptional activation of yeast genes was also shown to be mediated by enhancer-like sequences, known as upstream activation sequences (UASs), although their distances from the core promoters are much shorter (within a few hundred base pairs) than the typical distances between enhancers and promoters in mammals (Guarente, 1988). These results led to the realization that enhancer activity is the primary mechanism for determining the spatiotemporal gene expression pattern in eukaryotes.

RNAPII Association at Enhancers and Locus Control Regions

The ability to recruit RNAPII and initiate transcription has generally been considered the most unique property of promoters. However, even before the genomics era, several studies found that RNAPII can be directly recruited to enhancers upon transcriptional induction, potentially serving as a regulatory checkpoint for RNAPII delivery to the target promoter. Interestingly, an early study of the SV40 enhancer found that, in the absence of any known promoter sequence, the 72 bp DNA repeats can also "promote" gene expression, although this was deemed to be inefficient (Benoist and Chambon, 1981; Moreau et al., 1981). This finding suggested the possibility that the 72 bp sequence might serve as a general entry site for a component of the transcription machinery, such as RNAPII, which could then track along the DNA to a transcription initiation site (Moreau et al., 1981). Another proposed mechanism that may not be mutually exclusive with the RNAPII tracking model is the chromatin remodeling effect. As various chromatin modifying enzymes such as histone acetyltransferases and methyltransferases can be part of the RNAPII transcription complex (Cho et al., 1998; Gerber and Shilatifard, 2003), transcription initiated from the enhancer proceeding across the intervening regions between the enhancer and the target promoter might be responsible for establishment and/or maintenance of an active chromatin conformation required for efficient gene transcription.

Initial studies of enhancer identification and characterization were carried out by transient transfection experiments, which means that enhancer activity may be subject to position-effect variegation, depending on the chromatin configuration at the genomic site of integration. However, a study of a transgene containing the human β-globin locus discovered that five DNase-I-hypersensitive sites scattered in a ~70 kb region surrounding the β-globin gene were sufficient to overcome the positional effect (Grosveld et al., 1987). These *cis*-regulatory regions (e.g., enhancers) conferring activation of a linked gene in a tissue-specific, copy-number-dependent manner, independent of its position of integration, were collectively termed a locus control region (LCR) (Orkin, 1990). Notably, transcription activity was detected at enhancers located within the β-globin LCR region and throughout the intervening DNA into the globin genes (Ashe et al., 1997; Routledge and Proudfoot, 2002; Tuan et al., 1992). These LCR-driven intergenic transcripts are relatively short (< 3 kb), remain in discrete foci in the nucleus, and do not encode proteins (Ling et al., 2005). Transcription predominantly occurred toward the downstream globin genes but was independent of the orientation, position, and distance of the enhancers with respect to the gene (Kong et al., 1997; Routledge and Proudfoot, 2002). RNAPII recruitment and transcription activity have also been observed in other LCRs, including those that control expression of major histocompatibility complex (MHC) class II in antigen-presenting immune cells and pituitary-specific expression of the human growth hormone (*hGH*) gene (Ho et al., 2006; Masternak et al., 2003). Interestingly, insertion of an exogenous RNAPII termination sequence within the *hGH*-LCR blocked *hGH* regulation, suggesting that transcription through the LCR domain is a functionally important event.

In both the human and murine β-globin gene loci, RNAPII interacts with the LCR, but not directly with the β-globin gene prior to erythroid differentiation, whereas it is associated with both in differentiated erythroid cells (Levings et al., 2006; Vieira et al., 2004). In an in vitro assay using nuclear extracts from MEL cells, RNAPII and other basal transcription factors associated with immobilized LCR templates could be transferred to a β-globin gene template, which was facilitated by the erythroid transcription factor NF-E2 (Vieira et al., 2004). Although performed in vitro, these results suggest a model in which the β-globin LCR functions to assemble and hold the RNAPII transcription complex for timely delivery to the β-globin gene to ensure the developmentally stage-specific expression. Furthermore, blocking transcription elongation between the LCR and the promoter by inserting a transcription terminator sequence significantly decreased the β-globin mRNA level, suggesting that the β-globin LCR facilitates a tracking and transcription mechanism (Ling et al., 2004). A similar mechanism has been proposed for other LCRs and enhancers (Ho et al., 2006; Wang et al., 2005). In a contrasting model, transfer of the RNAPII machinery from the α-globin LCR to the promoter appears to be mediated by formation of a DNA loop between the LCR and the promoter, as no RNAPII signal is detected in the intervening DNA between the LCR and the promoter (Vernimmen et al., 2007).

Genome-wide Architecture of Enhancers

These initial insights into the complex roles of enhancers and LCRs set the stage for thinking about regulatory elements in a more global manner. Early genome-wide studies identified RNAPII binding at intergenic loci, which suggested the existence of enhancer-like sequences across the genome; however, there were questions regarding the functional relevance of such RNAPII occupancy (Barrera et al., 2008; Brodsky et al., 2005; Carroll et al., 2006; Heintzman et al., 2007). Moreover, it was difficult to classify RNAPII binding sites as possible enhancer or un-annotated promoter of a protein-coding gene by the virtue of RNAPII association alone.

It became clear that additional criteria would be needed to identify enhancers. Given their association with transcription factors, computational analysis of TF binding motifs combined with the assessment of evolutionary conservation within the DNA was used as a popular approach in identifying enhancers (reviewed in

Aerts, 2012). More recently, chromatin-immunoprecipitation-based analysis of TF binding in vivo (e.g., ChIP-chip and ChIP-seq) has been widely used to experimentally determine actual TF binding sites in vivo. This approach revealed that only a small fraction of TF binding motifs are actually bound by TFs in vivo in a given tissue and/or stage (ENCODE Project Consortium et al., 2007). TF binding per se does not signal a functional outcome. Functional activation requires recruitment of additional cofactors or mechanisms involving a combinatorial coordination of multiple TFs. Therefore, analysis of evolutionarily conserved TF motifs or TF binding alone has a limited power for identification and prediction of functional enhancers (see also Kellis et al., 2014 for review).

Transcriptional coactivators p300 and CBP interact with a large number of transcriptional activators and the general transcription machinery, including RNAPII. Moreover, both p300 and CBP display acetyltransferase activity toward the tails of histones localized near *cis*-regulatory regions, which is thought to create a transcriptionally permissive chromatin structure. Therefore, although not perfect, genome-wide analysis of p300/CBP binding sites has been commonly used as a method for identifying enhancer elements in vivo without having to investigate individual TFs (May et al., 2012; Visel et al., 2009)

A complementary approach in identifying enhancers takes advantage of their chromatin accessibility. The assembly of various TF complexes at *cis*-regulatory regions is considered to compete with stable association of nucleosomes. As a result, active enhancers and promoters have reduced nucleosome density and display hypersensitivity to DNase I digestion. This feature of chromatin accessibility has been utilized in next-generation sequencing-based techniques such DNase-seq, FAIRE-seq, and ATAC-seq (Boyle et al., 2008; Buenrostro et al., 2013; Giresi et al., 2007) to identify enhancers without any prior knowledge of TF binding motifs or TF binding. Although not sufficient to pinpoint cell-type-specific enhancers due to its indiscriminate nature, this method can be very useful for enhancer characterization when combined with other mapping techniques.

An increasing number of epigenomic studies have illustrated that the chromatin of metazoan genomes is organized into modular domains that represent unique chromatin states formed by a combination of multiple post-translational modifications on histones within the nucleosomes (ENCODE Project Consortium, 2012; Ernst et al., 2011). For example, nucleosomes within enhancer regions contain histone variants H3.3 and H2A.Z (Goldberg et al., 2010; Henikoff et al., 2009; Jin et al., 2009). These nucleosome variants are deposited into enhancer regions in a replication-independent manner and are more sensitive to high salt than canonical nucleosomes. In contrast, nucleosomes flanking TF-bound sites are stable and undergo various histone modifications that are distinctive to each functional domain and across cell types and also correlate with transcriptional outputs (ENCODE Project Consortium, 2012; Heintzman et al., 2007, 2009; Hon et al., 2009; Visel et al., 2009). Importantly, such chromatin modifications combined with other measures (chromatin accessibility and TF binding) have proven themselves a useful barometer for active enhancers. Enhancers of active genes generally display a high level of mono- or di-methylation on H3 lysine 4 (H3K4me1/2) but are low or devoid of H3K4me3, whereas promoter sequences show the opposite pattern. In addition to H3K4me1/2, mutually exclusive modifications on H3K27 residues co-segregate with active or inactive/poised enhancers (Creyghton et al., 2010; Rada-Iglesias et al., 2011). Active enhancers are enriched in the H3K27ac mark, a major substrate for the histone acetyltransferase p300/CBP (Jin et al., 2011; Tie et al., 2009), while poised enhancers are associated with H3K27me3, a mark enriched in Polycomb (PcG)-associated and transcriptionally repressed regions (Rada-Iglesias et al., 2011). Additionally, H3K27me3 also co-exists with the active promoter mark H3K4me3 in the promoters of developmentally silenced genes in ES cells, known as poised/bivalent promoters (Bernstein et al., 2006).

Although enhancers share common structural and functional features, as described above, individual enhancers widely differ in the enrichment levels of TF- and enhancer-specific histone modifications. A set of recent studies inspected enhancers based on the quantitative difference in the level of Mediator complex binding or H3K27ac marks and found that enhancers are often clustered in large domains, termed super-enhancers. Typically a few hundred super-enhancers are present in a given cell type and are often located near cell-type-specific genes or the genes that control the biological processes that define the identities of the cell types (Hnisz et al., 2013; Lovén et al., 2013; Whyte et al., 2013). Consistently, a strong enrichment of disease-associated non-coding variants has been observed within super-enhancers (Hnisz et al., 2013). Each super-enhancer represents a functional cluster of multiple enhancer units that communicate with each other physically and functionally and provide a platform where various signaling pathways converge to robustly regulate genes that control cell identity during development and tumorigenesis (Hnisz et al., 2015). With that operational definition, super-enhancers appear to be highly analogous to the "regulatory archipelago" described at the *HoxD* locus (Montavon et al., 2011). Although more analysis will be required to establish whether or not super-enhancers reflect a novel paradigm in gene regulation, their identification in each cell type would, at least, be very useful for the characterization of the cell-type-specific regulatory network.

From Enhancer Sequences to Enhancer RNAs

In 2010, two independent studies reported that direct RNAPII recruitment and transcription are genome-wide features of functionally active enhancers. In neurons, a combination of enhancer markers (high levels of H3K4me1 overlapped with CBP binding but with no or low H3K4me3) was used to identify ~12,000 neuronal enhancers that mediate transcription induction upon neuronal activation by membrane depolarization (Kim et al., 2010). Interestingly ~25% of the neuronal enhancers also exhibited a significant level of RNAPII binding and produced RNA transcripts. These enhancer RNAs (eRNAs) are dynamically regulated by neural activity, with their levels positively correlating with mRNA levels of nearby protein-coding genes. The majority of eRNAs characterized in neurons are short (<2 kb), lack polyadenylated tails, and do not appear to be spliced. Notably, global profiling showed that eRNAs are transcribed bi-directionally from the center of enhancers, where CBP and RNAPII are bound.

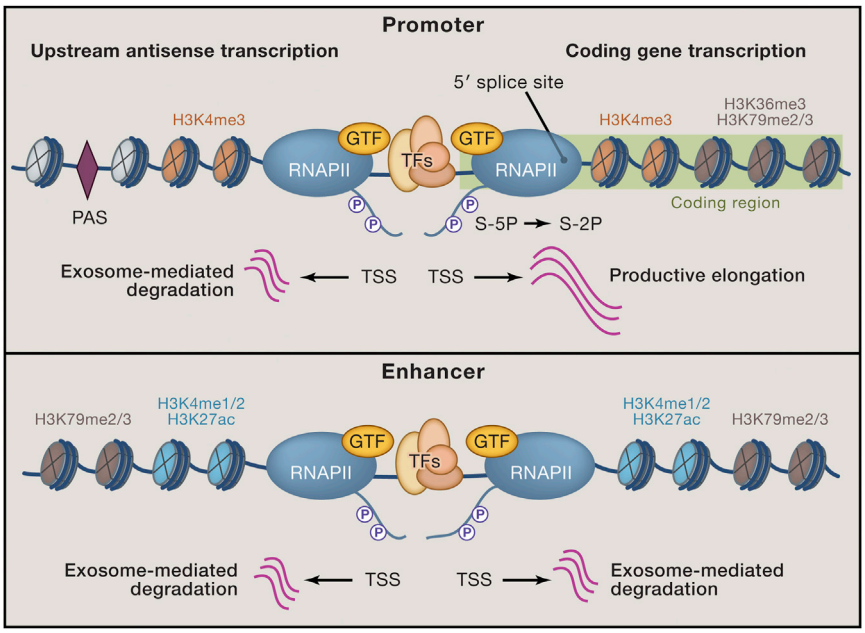

Figure 1. A Contemporary View on Promoters and Enhancers

Features of promoters include transcription initiation in the sense and anti-sense direction being mediated by the transcription machinery assembled independently onto its own core promoter. Although not shown here, convergent transcription has been observed at the promoters of weakly expressed genes. H3K4me3 is highly enriched at the promoter regions. Enhancer-like chromatin signatures (H3K4me1 and H3K27ac) and the Tyr-1P form of RNAPII have also been observed near the upstream anti-sense TSSs. Polyadenylation sites are enriched near the 3′ end of the upstream anti-sense RNAs and mediate the exosome-dependent degradation of the antisense RNAs. 5′ splice sites are only present in the coding gene and might contribute to the productive elongation of sense mRNA transcripts through the binding of the U1 splicing complex, which blocks PAS-mediated early termination. The Ser-5P form of RNAPII is engaged in upstream anti-sense transcription, but it is not known whether Ser-2P of RNAPII occurs during the elongation of anti-sense RNA.

Features of enhancers include, as with the promoter, recruitment of the general transcription factors (GTF), including RNAPII, and initiation of transcription at defined sites. Enhancer-driven transcription typically exhibits more prominent bi-directionality than that stemming from the promoter. H3K4me1/2 is commonly enriched at enhancers. Functionally active enhancers also exhibit a high level of H3K27 acetylation, whereas poised or inactive enhancers are marked by H3K27me3. Ser-5P and Tyr-1P forms of the RNAPII have been observed. It is not clear whether or not Ser-2P RNAPII and H3K36me3 marks are present at active enhancers. 5′ splice site sequences are not enriched near the regions surrounding enhancers. Both strands of enhancer RNAs appear to be degraded by the exosome, although it is not known whether it is mediated by the PAS-dependent mechanism.

Another study discovered eRNAs (originally referred to in the study as inducible upstream extragenic transcripts) in endotoxin-stimulated primary macrophages (De Santa et al., 2010). RNAPII ChIP-seq analysis identified 4,855 extragenic RNAPII binding sites, and ~70% of them showed an enhancer-like chromatin signature (high levels of H3K4me1 with low or no H3K4me3). Many of these extragenic enhancers produce eRNAs upon LPS stimulation. Unlike neuronal eRNAs, several macrophage eRNAs were shown to be produced from uni-directional transcription and to be polyadenylated without being spliced.

Since these initial discoveries, eRNAs have been found in many mammalian cell types, including embryonic stem cells, suggesting that eRNA synthesis is a universal cellular mechanism (reviewed in Lam et al., 2014). Super-enhancers exhibit a much higher level of RNAPII binding and eRNA transcription than typical enhancers (Hah et al., 2015; Hnisz et al., 2013). Multiple eRNAs are generated within super-enhancers with a striking correlation in their expression patterns, which could imply that each super-enhancer might form a single regulatory module (Hah et al., 2015). Importantly, a recent study has identified the RNAPII-associated complex, Integrator, as the molecular machine involved in the 3′ end processing of eRNAs at enhancers and super-enhancers (Lai et al., 2015).

As greater numbers of eRNAs have been identified, we've gained more detailed insights into their properties and regulation. The majority, although not all, of eRNAs in the nucleus lack polyadenylated tails (ENCODE Project Consortium, 2012; Derrien et al., 2012; Djebali et al., 2012; Harrow et al., 2012). A genome-wide study in murine CD4+ CD8+ thymocytes correlated non-polyadenylated and polyadenylated eRNAs with bi-directional and uni-directional transcription, respectively, although the functional implication of this dichotomy is not known (Koch et al., 2011; Natoli and Andrau, 2012). Moreover, eRNA-producing enhancers are cell-type specific and are associated with a chromatin signature unique to functionally active enhancers, including H3K4me1, H3K27 acetylation, and H3K79 dimethylation, along with RNAPII binding (Djebali et al., 2012). A genome-wide chromosomal interaction study in several human cell lines further demonstrated that eRNA-producing enhancers are preferentially engaged in an interaction with the proximal promoters (Sanyal et al., 2012). Another notable feature of eRNAs is the timing of their expression relative to mRNA upon stimulus-dependent induction. In many different cell types, eRNA transcription marks the earliest response in the wave of transcriptional change when cells undergo a state change in response to environmental or developmental cues (Arner et al., 2015; De Santa et al., 2010; Hah et al., 2013; Hsieh et al., 2014; Schaukowitch et al., 2014).

Promoter versus Enhancer: A New Comparison in the Genomic Era

Recent genome-wide evidence of transcribing enhancers observed in a wide range of cell types argues that the conventional definitions of the promoter and the enhancer must be revised. The roles of promoters and enhancers in transcription have been thought to be distinct; however, these two regulatory elements are highly interrelated and show noticeable similarities in structure and function. As summarized below, both the promoter and the enhancer exhibit common structural and functional features that have not been previously appreciated (Figure 1).

Shared Local Structure

Both promoters and enhancers display DNase I hypersensitivity, which results from depletion of nucleosomes. This local structure arises because both regulatory regions are composed of binding sites for TFs, which exclude nucleosomes. However, whether or not there is any distinguishable difference in TF binding site composition between the regions is somewhat debatable. Initial genome-wide studies suggested that promoters and enhancers differ in the composition of binding sites (Rada-Iglesias et al., 2011; Shen et al., 2012; Thurman et al., 2012). However, recent FANTOM5 cap analysis gene expression (CAGE) studies argue that the difference in binding site composition might simply result from the fact that enhancers are largely devoid of CpG islands (CGI) and repeats resembling non-CGI promoters (Andersson et al., 2014). Consistently, some older studies showed that interacting promoter-enhancer pairs often harbor common TF binding sites (Bienz and Pelham, 1986; Bohmann et al., 1987; Parslow et al., 1987).

Consistent Histone Modification Patterns

Although the local ratio of H3K4me3/me1 has been widely used as a means to distinguish enhancers and promoters, recent studies argue that the three H3K4 methylation states (H3K4me1/2/3) simply reflect dynamic changes in transcription activities of both the promoters and enhancers rather than representing static and intrinsic features of individual regulatory elements. The majority of enhancers simply show a low H3K4me3/me1 ratio, as their transcription level is generally lower than promoter-driven transcription. However, H3K4me3 is often observed at functionally active enhancers, and the H3K4me3/me1 ratio positively correlates with transcription level, independent of transcript stability (Core et al., 2014; Koch et al., 2011; Pekowska et al., 2011).

Functional Interchangeability

The notion that promoters and enhancers functionally overlap was initially supported by the finding that the proximal promoter region of mouse metallothionein I (*Mt1*) gene, when inserted downstream of a rabbit β-globin test gene, could enhance β-globin transcription upon metal ion stimulation, thus acting as an inducible enhancer (Serfling et al., 1985). Moreover, a chromosomal interaction study found that promoters frequently associate with other promoters through space analogous to well-characterized promoter-enhancer interactions, which could imply an enhancer-like function of the promoter in transcription (Li et al., 2012). Recently, intragenic enhancers were shown to frequently function as alternative tissue-specific promoters, producing a class of abundant, spliced, multi-exonic poly(A)$^+$ RNAs (meRNAs) reflecting the host gene's structure (Kowalczyk et al., 2012). These examples collectively support the notion that the enhancers and promoters not only share many of the similar architectural features (nucleosome hypersensitivity and chromatin marks), but also may be functionally interchangeable.

Common Mechanisms to Control RNA Synthesis

Similar to promoters, RNAPII and general transcription factors (GTFs) are assembled on enhancers and initiate transcription (Koch et al., 2011; Natoli and Andrau, 2012). The C-terminal domain (CTD) of RNAPII is composed of multiple heptapeptide repeats (YSPTSPS) and undergoes differential phosphorylation as the transcription cycle progresses. While unphosphorylated RNAPII enters the pre-initiation complex, escape from the promoter is highlighted by phosphorylation of the Ser-5 residues of the CTD, and entry of the RNAPII into productive elongation is coordinated by a wave of Ser-2 phosphorylation. Both unphosphorylated and Ser-5-phosphorylated forms of RNAPII are also observed at enhancers. Moreover, tyrosine 1 phosphorylation of the RNAPII CTD has been observed with antisense promoter transcription and active enhancers in mammalian cells (Descostes et al., 2014). However, the elongation-specific form of RNAPII (Ser-2-phosphorylated), as well as the H3K36me3 mark, both of which are normally seen across the coding regions of actively transcribing genes, have not been readily detected in the eRNA transcribing areas (Kaikkonen et al., 2013; Koch et al., 2011; Natoli and Andrau, 2012). On the other hand, several studies observed H3K79me2/3 marks over transcribed enhancer regions, an additional coding-region-specific modification whose levels are highly correlated with transcription activity (Bonn et al., 2012; Djebali et al., 2012).

At this point, it is not clear whether the lack of elongation-specific marks (H3K36me3 and Ser-2 phosphorylation of RNAPII) at enhancers reflect a fundamentally different transcription mechanism between enhancers and promoters or whether the eRNA regions being transcribed are simply not long enough to sufficiently accumulate those marks, which are known to be enriched near the 3′ end of genes. Alternatively, the levels of eRNA transcription might not be high enough to observe the enrichment of these elongation-specific features, as their levels are generally correlated with transcription output.

Bi-Directional Transcription

Bi-directionality is a striking feature of eRNA transcription that has been documented at many enhancers. However, the majority of mammalian promoters also drive divergent transcription, resulting in the production of short antisense ncRNAs (known as uaRNAs, PROMPTs, or promoter upstream transcripts) from upstream promoter regions in addition to sense mRNAs (Core et al., 2008; Preker et al., 2008; Seila et al., 2008). Both eRNAs and promoter upstream antisense transcripts are relatively unstable, possibly due to exosome-mediated degradation (Andersson et al., 2014; Flynn et al., 2011). Genome-wide analyses integrating nascent transcript mapping, DNase I hypersensitive sites, nucleosome positions, and binding profiles of various TFs and histone modifications have corroborated the shared architecture of transcription initiation between enhancers and promoters. Both enhancers and promoters exhibit similar frequencies of canonical core promoter elements, highly positioned flanking nucleosomes, and tight average spacing (~110 bp) between each pair of divergent TSSs. Divergent transcription at promoters and enhancers is mediated by independent RNAPII transcription complexes assembled at each TSSs, which is intrinsically configured by underlying core elements as well as TF binding motifs enriched near both sense and anti-sense TSSs (Core et al., 2014; Duttke et al., 2015; Scruggs et al., 2015). Moreover, elevated levels of TF binding and enhancer-like chromatin signatures (e.g., high levels of H3K4me1 and H3K27ac) were observed near the anti-sense TSSs located upstream of highly transcribed sense TSSs (Scruggs et al., 2015). Intriguingly, a nucleotide-resolution mapping analysis of

RNAPII position by native elongating transcript sequencing (NET-seq) has revealed that the promoters of genes expressed at a low level in human HeLa or HEK293T cells drive convergent transcription, in which antisense transcription originates downstream of the sense TSS (Mayer et al., 2015). It is not known whether convergent transcription is also a feature of enhancers.

Regulation of Upstream Transcription

Computational analysis of promoters showed that the regions where upstream antisense transcription occurs are enriched in polyadenylation sites (PAS) but are depleted of potential U1 small nuclear ribonucleoprotein (snRNP) recognition sites, or 5′ splice site-like sequences. This asymmetric feature in functional DNA motifs flanking TSSs was argued to underlie promoter directionality (Almada et al., 2013; Core et al., 2014; Ntini et al., 2013). Transcription of upstream antisense RNAs terminates at the enriched PAS, and the RNAs are then degraded by the exosome, whereas the sense transcripts are protected by U1 snRNP, which prevents premature cleavage and polyadenylation (Berg et al., 2012; Kaida et al., 2010). The FANTOM5 CAGE analysis suggested that the eRNAs are also subject to a similar decay mechanism. However, unlike the promoters, the DNA regions flanking enhancers do not show an enrichment of 5′ splice site sequences (Andersson et al., 2014).

The Role of Enhancer Transcription

The defined characteristics of eRNAs—low abundance, low stability, lack of RNA processing such as polyadenylation and splicing, and bi-directionality in transcription—could collectively suggest that eRNAs are the byproduct of enhancer transcription activity, with no biological function. This idea of transcriptional noise proposes that excess RNAPII machinery is uniformly associated with physically accessible genomic regions, including enhancer regions, and initiates transcription "nonspecifically" from incorrect sites (Struhl, 2007). In this model, nonspecific transcripts are generally in low abundance, as they are rapidly degraded by intrinsic cellular surveillance mechanisms such as nonsense-mediated decay or exosome-mediated degradation (LaCava et al., 2005; Wyers et al., 2005).

However, transcription does not appear to be a random process. For example, there is no transcription activity in poised enhancers, which clearly show chromatin accessibility judged by DNase I hypersensitivity. It was also proposed that enhancers that mediate rapid induction of neural genes in response to membrane depolarization do not transcribe eRNAs unless the enhancer is paired with its target promoter (Kim et al., 2010). However, enhancer transcription initiated from *hGH*-LCR in the pituitary was independent of the interaction with the target *hGH*-N promoter (Yoo et al., 2012). Despite this discrepancy in the promoter dependency of eRNA production, it is generally agreed that eRNA transcription occurs only from functionally active enhancers in a regulated manner (Andersson et al., 2014; Core et al., 2014; Creyghton et al., 2010; Hah et al., 2011; Kaikkonen et al., 2013; Kim et al., 2010; Rada-Iglesias et al., 2011). Furthermore, as we have described, both enhancers and promoters share key architectures of transcriptional initiation sites. These features collectively suggest that eRNA synthesis is a regulated process, with its transcription initiation fidelity comparable to the promoter, rather than a consequence of random RNAPII transcription initiation from accessible genomic regions. However, they do not necessarily prove the functionality of eRNA transcripts (Weingarten-Gabbay and Segal, 2014).

When considering the functional relevance of enhancer transcription, several lines of evidence suggest that the act of eRNA transcription, rather than the eRNA transcript itself, might have a specific biological function. One possibility is that enhancer-promoter pairing or looping is mediated by a tracking mechanism in which the enhancer-bound transcription complex is ferried to a specific target promoter via uni-directional RNAPII transcription. Consistently, LCR-driven transcription takes a uni-directional path toward target genes (Ashe et al., 1997; Ho et al., 2006; Ling et al., 2005; Routledge and Proudfoot, 2002), and some eRNAs in T lymphocytes were also shown to be transcribed uni-directionally (Koch et al., 2011; Natoli and Andrau, 2012). However, global profiles of eRNA expression argue that such a simple tracking/scanning mechanism of enhancer-promoter communication might not be general, as the majority of enhancer transcription occurs bi-directionally within confined flanking regions not contiguous to the target gene.

Since RNAPII can carry histone-modifying enzymes through interactions with its CTD (see review in Selth et al., 2010), RNAPII transcription could be an underlying mechanism for altering the chromatin architecture at enhancers or intervening DNA regions between enhancers and promoters. Indeed, active chromatin modifications such as histone hyperacetylation and DNase I hypersensitivity are often observed near RNAPII-transcribed regions (Bulger et al., 2003; Gribnau et al., 2000; Masternak et al., 2003; Travers, 1999). For example, a transcription inhibitor, actinomycin D, significantly blocked LPS-induced histone hyperacetylation in the intervening regions between inducible gene promoters and enhancers in macrophages (De Santa et al., 2010). Another study in macrophages showed that TLR4-signaling-induced eRNA transcription precedes a local increase in the level of H3K4me1/2, and the length of eRNAs coincides with the width of the H3K4me1/2-modified region (Kaikkonen et al., 2013). A transcription elongation inhibitor, flavopiridol, but not eRNA knockdown, significantly reduces the level of H3K4me1/2 at enhancers, suggesting that transcription activity at enhancers, not the eRNA transcript itself, might be important for at least some aspect of enhancer-specific chromatin modification (Kaikkonen et al., 2013). However, flavopiridol treatment in MCF-7 cells did not alter the levels of enhancer-specific histone marks (i.e., H3K4me1 or H3K27ac) (Hah et al., 2013). One potential source for this discrepancy might be differences in the stability of the enhancer-specific marks between the two cell types (T cells versus MCF-7 cells) and/or the mode of stimulus-induced signaling, although the aforementioned study in macrophages claimed that the effect of transcription blockers in H3K4me1/2 modifications is also observed in pre-existing enhancers (Kaikkonen et al., 2013). It also needs to be noted that the proposed function of enhancer transcription in the enhancer-specific chromatin landscape does not have to be mutually exclusive with the possibility that the eRNA transcript itself might play a functional role in transcriptional activation.

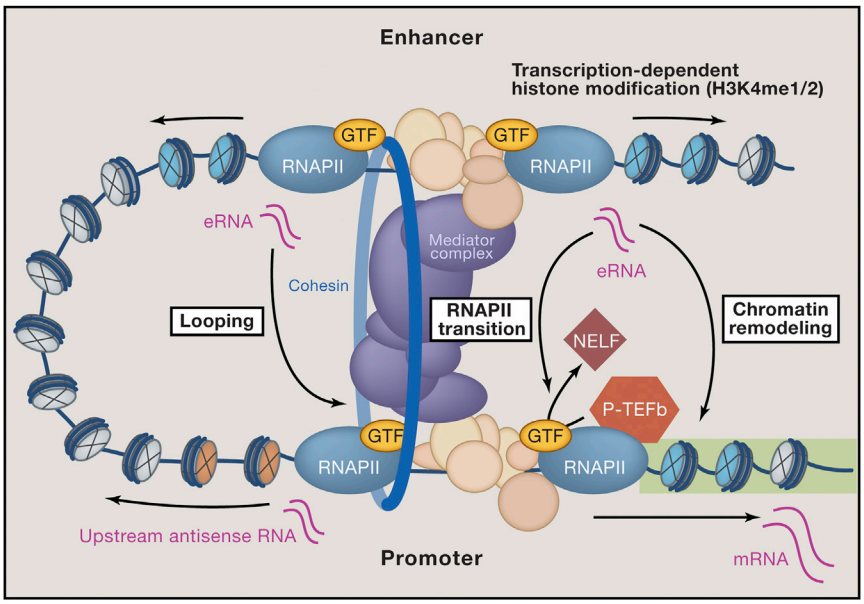

Figure 2. Mechanisms of Enhancer-Promoter Interactions

H3K4me1/2 modification at enhancers can be mediated by RNAPII transcription activity. Enhancer RNA is also shown to play a role in various stages of transcription. Looping: The Mediator/Cohesin complex is involved in stable formation of enhancer-promoter looping. Some eRNAs (e.g., ncRNA-a and eRNAs expressed from estrogen receptor-α bound enhancers) facilitate the looping through an interaction with the subunit(s) of the Mediator/Cohesin complex. Chromatin remodeling: eRNAs (e.g., *^{CE}RNA*) can also promote transcription by remodeling the chromatin structure such that the accessibility of RNAPII machinery is increased. RNAPII transition: Early RNAPII elongation is another transcription step regulated by eRNAs. eRNAs (e.g., *Arc* eRNAs) can help RNAPII enter into a productive elongation stage by facilitating transient release of the negative elongation factor, NELF, which causes RNAPII pausing near the TSS.

The Role of eRNA Transcript

Several recent studies have suggested that the eRNA transcript itself might have an activating role in target gene expression in various cell types (Hsieh et al., 2014; Lam et al., 2013; Li et al., 2013; Melo et al., 2012; Mousavi et al., 2013; Ilott et al., 2014; Schaukowitch et al., 2014). Knockdown of eRNAs generated from various enhancer regions consistently causes a decrease in the expression of their specific target genes. Multiple mechanisms have been described to underlie the eRNA function. These include the eRNAs regulating enhancer-promoter looping, chromatin remodeling, and early transcription elongation (Figure 2). In human MCF-7 breast cancer cells, several eRNAs expressed from estrogen receptor-α-bound enhancers facilitate specific enhancer-promoter interactions in a ligand-dependent manner by recruiting the cohesin complex to the enhancer from which they originated (Li et al., 2013). An eRNA expressed from Kallikrein-related peptidase 3 (*KLK3*) enhancer, one of the strongest androgen receptor (AR)-bound enhancers in prostate cancer cells, was also shown to facilitate a specific interaction between the *KLK3* enhancer and the *KLK2* promoter but in this case by forming a complex with AR and a subunit of the Mediator complex, MED1 (Hsieh et al., 2014). Therefore, chromosomal looping between specific set(s) of enhancers and promoters appears to be a key regulatory step in which both eRNAs and other activating lncRNAs can commonly act (Figure 2). By contrast, the eRNA expressed from the *MYOD1* core enhancer (*^{CE}RNA*) during the myogenic differentiation of C2C12 skeletal muscle cells had no impact on the enhancer-promoter interaction (Mousavi et al., 2013). Instead, *^{CE}RNA* increased RNAPII occupancy at the promoter region of the *MYOD1* gene and subsequent transcription by promoting chromatin accessibility. Although the exact mechanism has not been defined, the chromatin remodeling activity of *^{CE}RNA* is reminiscent of the function of *HOTTIP* (Wang et al., 2011) (Figure 2).

Early transcription elongation is another step in which eRNAs play a role. RNAPII pausing immediately downstream of the transcription start sites is a widespread regulatory mechanism in higher eukaryotes, which is mediated by negative elongation factor, NELF, and DRB sensitivity-inducing factor. By serving as a key rate-limiting step, RNAPII pausing allows the convergence of signaling pathways and is thought to be important for the establishment of permissible chromatin structure as well as rapid and/or synchronous gene expression (Adelman and Lis, 2012). During induction of neuronal immediate early genes, eRNAs contribute to the gene induction in *cis* by promoting efficient release of NELF from their target gene promoters. eRNAs are rapidly transcribed and destabilize NELF's association with paused RNAPII by directly binding to the RNA recognition motif present in the NELF-E subunit (Schaukowitch et al., 2014). Knockdown of eRNA blocks transient release of NELF from the promoter during transcription activation and specifically decreases the amount of elongating RNAPII without affecting the RNAPII recruitment step or chromosomal looping between the enhancer and the promoter (Figure 2).

lncRNAs with Enhancer-like Functions

In parallel with the eRNA studies, an independent study discovered an enhancer-like function for a set of long non-coding RNAs (lncRNAs) in human cell lines, termed ncRNA-activating (ncRNA-a) (Lai et al., 2013; Ørom et al., 2010). Knockdown of several lncRNAs in this class invariably reduced expression levels of nearby protein coding genes. A subsequent mechanistic study revealed that the ncRNA-a recruits a transcription coactivator complex, Mediator, to facilitate chromosomal interaction between the ncRNA-a loci and its targets (Figure 2) (Lai et al., 2013). Mediator forms a complex with cohesin that creates a ring-like structure to keep two DNA segments together, which then regulates gene expression by connecting the enhancers and promoters of active genes in a cell-type-specific manner

(Kagey et al., 2010). In parallel, ncRNA-a stimulates the CDK8 kinase activity of Mediator to increase the level of histone H3 phosphorylation at serine 10 (H3S10), which is a mark associated with active chromatin and gene induction (Nowak and Corces, 2004).

Other lncRNAs also show related functions in different biological contexts. A Notch-regulated lncRNA, *LUNAR1* (*leukemia-induced noncoding activator RNA*), enhances *IGF1R* mRNA expression by a mechanism similar to ncRNA-a (Trimarchi et al., 2014). Importantly, the enhancer-like activity of *LUNAR1* for *IGF1R* expression was critical for the growth of T cell acute lymphoblastic leukemia cells both in vitro and in vivo. *HOTTIP* is a lncRNA expressed from the tip of the *HOXA* locus that coordinates the activation of several *HOXA* genes in vivo (Wang et al., 2011). Knockdown of *HOTTIP* specifically decreases expression of distally located *HOXA* genes, but not the highly homologous *HOXD* genes, which suggests a *cis* mechanism. Unlike ncRNA-a, *HOTTIP* does not affect the chromosomal interaction. Instead, chromosomal looping brings *HOTTIP* into close proximity to the *HOXA* gene locus where *HOTTIP* promotes histone H3 lysine 4 trimethylation and gene transcription by recruiting WDR5/MLL methyltransferase complexes. *NeST* (nettoie Salmonella pas Theiler's [cleanup Salmonella not Theiler's]) is another enhancer-like lncRNA that works together with WDR5 to increase H3K4me3 level at the interferon-γ (*Ifng*) gene in activated T cells. Transgenic overexpression of *NeST* was shown to induce IFN-γ synthesis in activated CD8+ T cells, suggesting a possible *trans*-mechanism to regulate its neighboring gene. Interestingly, a recent study found the previously described lncRNA, ncRNA-a3, mapping to a bi-directionally transcribed enhancer of the *TAL1* gene (Ørom et al., 2010; Vučićević et al., 2015). Therefore, it is likely that, as eRNAs in different human cells are fully cataloged, many of the currently annotated lncRNAs with enhancer-like function will fall under the classification of eRNAs (Vučićević et al., 2015).

Prospects

Transcription activity at enhancers was first hinted by the promoter-like activity (i.e., able to initiate transcription) of the first viral enhancer, the 72 bp tandem DNA repeats located upstream of SV40 early gene. Subsequently, several cellular LCRs and enhancers were also shown to transcribe ncRNAs. Nonetheless, transcriptional activity was not regarded as a general feature of enhancers until the advent of genome-wide studies. It now seems clear that ncRNA transcription is a signature of functionally active enhancers at least in higher metazoans.

As described above, some experimental evidence already supports the roles of both enhancer transcription and the eRNA transcript in gene expression. However, we are still far from fully understanding the functional and biological significance of eRNAs, and more thorough studies on eRNA function and mechanism will be required. For example, the molecular determinants of eRNA function have not been studied, and thus it is not known whether specific sequences or secondary structures would be critical for eRNA function. Moreover, although some studies found that only the sense eRNAs—transcribed in the same direction with its target mRNA—appear to be sufficient for the eRNA function (Lam et al., 2013; Li et al., 2013), it is not clear at this point whether strand-specific functionality is a general feature of eRNAs. It also needs to be mentioned that all current functional studies of eRNAs have relied exclusively on knockdown and/or overexpression approaches in cell culture; hence, in vivo relevance is yet to be validated. While in vitro analytical methods offer technical advantages in mechanistic studies, several recent examples show that the findings from cell line studies in vitro are not observed or are quite different in knockout animals (Kohtz, 2014). Therefore, determining the biological significance of eRNAs in an in vivo context is imperative.

The functionality question aside, widespread observation of transcribed enhancers across multiple mammalian cell types calls for revising the traditional definition of "promoters" as the DNA regions that allow accurate transcription initiation of a gene. Similar to a promoter, an enhancer can direct RNA transcription from a defined site by independent RNAPII transcription machinery assembled with general TFs. Initiation of bi-directional transcription is another shared feature of transcriptional regulatory elements. Moreover, many of the features of upstream anti-sense transcripts mirror those of eRNAs, including their inherent instability and their enrichment of the tyrosine 1 phosphorylated form of RNAPII.

Importantly, the distinctive characteristic of the promoters is their ability to direct transcription of a spliced, polyadenylated transcript. In contrast to the promoter-driven mRNAs, eRNAs and upstream anti-sense RNAs are shorter in length (a few hundred base pairs up to a few kilobases) and by and large less stable. In addition, they are commonly subject to early termination through the action of the Integrator complex, which is consistent with their lack of 5′ splice sites and polyadenylation-dependent cleavage. However, as far as transcription initiation is concerned, there appears to be very little difference between the promoter and the enhancer. Indeed, in many examples, enhancers may look reminiscent of weak promoters transcribing low levels of RNAs. Additional studies will certainly be needed before we can fully understand and define the structural and functional identities of enhancers and promoters and their interrelationship. Nonetheless, the recent unveiling of shared transcriptional architectures between the two regulatory domains compels us to revise our old ways of thinking and incorporate new models of transcriptional regulation in eukaryotes.

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