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POINT-OF-VIEW

Transcriptional enhancers: Transcription, function and flexibility

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ABSTRACT
Active transcriptional enhancers are often transcribed to eRNAs, whose changing levels mirror those of the target gene mRNA. We discuss some of the reported functions of these eRNAs and their likely diversity to allow utilization of distinct *cis* regulatory regions to enhance transcription in diverse developmental and cellular contexts.

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Introduction

Transcriptional enhancers have traditionally been described as regulatory regions of DNA that elevate basal levels of transcription by increasing activity of the proximal promoter. This activity may involve binding of cell-specific transcription factors which, as a result of DNA looping, interact with and stabilize the general transcription machinery to direct the tissue-specific expression of a particular gene. However, a number of genome-wide sequencing projects have revealed that enhancers are often transcribed to long non-coding RNA (lncRNA) or enhancer RNA (eRNA), whose changes in levels frequently correlate with those of the target gene mRNA. Recent evidence suggests that these eRNAs may mediate crucial aspects of enhancer function, particularly by facilitating DNA looping and/or modification of the chromatin, although the mechanisms for this are largely still obscure.[1-4] Enhancer function is often highly cell context-dependent and distinct enhancer elements may be utilized very differently in diverse cell types, developmental stages or in response to external stimuli.[5] This essentially increases the possible modes of regulation of a particular gene, although it is not clear whether these diverse eRNAs have highly similar or perhaps distinct roles, depending on their particular genomic and cellular context. Moreover, the fact that a specific DNA element that acts as an enhancer can also sometimes drive transcription has led to a blurring in the definition of these two kinds of regulatory element.[6,7]

Transcription: Enhancer and eRNA structure

High-throughput sequencing studies in the last few years have demonstrated that enhancers are often enriched with RNAPII and are commonly transcribed. Typically, though not universally, these enhancer RNAs (eRNAs) are limited to around 800-2000 bp and arise from bi-directional transcription of both strands of DNA from a central non-transcribed region. Furthermore they are usually unspliced, only weakly expressed and lack polyadenylated tails suggesting very short half-lives.[1-3] Evidence suggests that some of these features may relate to the sequence at the 5'ends of the eRNAs, which, unlike the transcripts of protein-coding genes, often contain putative polyadenylation sites but lack binding sites for U1 small nuclear ribonucleic proteins, which appear to block early termination; the eRNAs are therefore not protected from cleavage and exosome-mediated degradation.[6,7]

Apart from these differences in the sequence, the chromatin landscape around the transcriptional start sites (TSSs) of enhancers also usually differs from that seen at proximal promoters[1-4] and this too may be responsible for some for the peculiar characteristics of the eRNA transcripts. Enhancers are typically enriched with H3K4me1 rather than the trimethylation

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(H3K4me3) commonly seen at promoters of protein-coding genes, the reasons for which are not clear. It was suggested that this merely reflects distinct rates of transcription, as H3K4me3 is also occasionally seen at enhancers and H3K4me1 sometimes at protein-coding gene start-sites.[6,7] However, protein-coding genes that are transcribed at low rates do not necessarily have higher H3K4me1 to K4me3 ratios.[8] Enhancers also frequently have particularly high levels of H3K27 acetylation, which is a mark of active chromatin, and may also play a role in the bi-directionality. At the start-sites of protein-coding genes, high levels of histone acetylation are correlated with bi-directional transcription, which is usually quickly terminated in the antisense direction.[9] The deacetylation-mediated repression of this antisense transcription in yeast reportedly involves recruitment of Set2 by phospho-Ser2 RNAPII (pS2-RNAPII), leading to trimethylation of H3K36 (H3K36me3); the histone deacetylase (HDAC), Rpd3S, is then recruited by H3K36me3.[9] Notably, some enhancers are not particularly enriched with pS2-RNAPII and H3K36me3,[1,10,11] which might prevent the recruitment of HDACs to enhancers through a similar mechanism, resulting in predominantly bi-directional transcription that is not so rapidly terminated due to the sequence elements mentioned above. A very recent publication also suggested a role for WDR82 in limiting these transcripts through both Set1 and protein phosphatase 1, both of which appear to play a role in terminating transcription of some lncRNAs.[12]

It is feasible that the genomic and epigenetic landscape at enhancers may also result in different histone modifications, due to the RNAPII being recruited to these elements via a variant pre-initiation complex (PIC). TFIID and other general transcription factors were seen to be recruited differently to enhancers and promoters,[10] and alternative compositions of PIC complexes were seen to be required for cell type- and gene-specific transcription, and are present in single cells of various tissues.[5] Different histone modifying enzymes in such variant complexes, as compared to those associated with the canonical PIC, could clearly also contribute to the divergent patterns of transcription described above.

Function: The role of the eRNA in enhancer function

It was initially unclear whether the weakly expressed and short-lived eRNAs have a function, and was suggested that they might be by-products of the proximity of these regions of DNA with active proximal promoters. However, escalating evidence indicates that eRNAs often do play a central role in the activity of the enhancer and their role in promoting effects on the transcription initiation complex and/or on chromatin structure of the target gene has been shown in a number of recent reports.[2] These include our own study on the eRNA transcribed from a distal enhancer of the pituitary gonadotropin *Cga* gene, which encodes the α-subunit common to the gonadotropin and thyrotropin hormones.[13] This enhancer was previously shown to drive the cell-specific expression of the *Cga* gene to the gonadotropes and thyrotropes,[14] and we demonstrated that the eRNA plays a pivotal role in transcription of this gene by facilitating the chromatin modifications that keep the proximal promoter, as well as the enhancer itself, in an active state. This function appears to arise from a role for the eRNA in facilitating the looping of the DNA between the enhancer and the proximal promoter, which was abolished following eRNA knockdown.[13]

Other studies have also demonstrated a role for lncRNAs in DNA looping, and enhancer interaction with gene promoters was noted to be greater for those enhancers that are transcribed than those that are not,[15] while these interactions tend to correlate with the levels of eRNA and target gene mRNA.[2-4] A role for eRNAs in DNA looping was shown also specifically for estrogen receptor α (ERα) and androgen receptor (AR)-regulated genes, and it was proposed that these eRNAs mediate the effects of the respective steroids on the promoter-enhancer interactions, through induction of this looping.[16,17] Also in studies in which the Integrator complex was depleted, which led to a reduction in stimulus-induced eRNA, DNA looping was affected adversely,[18] suggesting that DNA looping commonly requires presence of the eRNA.

Mechanistically, these observations are clarified somewhat by the finding that eRNAs interact with several components of the cohesion complex, which regulates enhancer-promoter interactions in stem cells.[16] By creating a ring-like structure around two distant genomic regions of DNA, cohesin facilitates their physical and thus also functional interaction. Various eRNAs and lncRNAs were shown to interact with the Mediator complex which augments this process by binding cohesin,[19] and this appears to play a crucial role in many enhancer-promoter

interactions.[2,3] In addition, a recent study showed that the architectural-regulator condensin complexes are recruited by ERα to active enhancers and that they regulate ligand-dependent enhancer activation by modulating the binding of various coactivators and corepressors. This facilitates DNA looping as well as eRNA transcription and target gene expression, although it was not determined whether their role also involves regulating higher order chromatin structure directly.[20]

Through induction of DNA looping, the eRNA of the *Cga* distal enhancer was seen to play a crucial role also in maintaining a state of euchromatin at the enhancer and proximal promoter, and knockdown of the eRNA dramatically altered the histone modifications at both sites. Most notable was the fact that both enhancer and promoter became associated with higher levels of histone H3, while H3K4me3 on the promoter was lost, and acetylation of H3K27 at the enhancer appeared to be replaced with repressive trimethylation of this residue.[13] Other studies have also reported that eRNAs or eRNA-like lncRNAs affect chromatin accessibility and RNAPII association with the target promoters (e.g., refs. 17, 21), and a more limited number of studies have reported an effect specifically on histone modifications (for example, ncRNA-a regulates the CDK8 kinase activity of mediator complex, which targets H3S10, and HOTTIP promotes H3K4me3 by recruiting WDR5/MLL methyltransferase complexes to the HOXA gene locus).[19,22] However, such specific actions would unlikely be relevant to all genes, as these histone modifications can be directed by alternative pathways and mechanisms in distinct genomic settings.

Some eRNAs appear to play a role also in transcriptional elongation. The induction of neuronal immediate early genes was seen to depend on the ability of eRNAs to induce release of the negative elongation factor (NELF) from paused RNAPII at their target gene promoters, without affecting RNAPII recruitment or DNA looping.[23] This pausing by RNAPII is thought to allow rapid and synchronous gene expression, while also first enabling the establishment of a suitable chromatin environment for transcription to proceed. RNAP pausing is common in many, though not all, mammalian genes, notable particularly at genes induced by specific signals,[24] suggesting that this function of the eRNA is likely also highly context-specific. Another recent study suggested that eRNAs might help maintain certain RNA/DNA binding transcription factors at regulatory elements.[25]

Flexibility in enhancer usage

Enhancers are often transcribed in cell-specific manner and may be highly responsive to the state of the cell.[26,27] The *Cga* eRNA levels were seen to reflect basal levels of promoter activity, in accordance with the report that this enhancer facilitates basal and tissue-specific expression of the *Cga* gene.[13,14] This function may relate to the binding of both enhancer and proximal promoter by the lineage-specific transcription factor Pitx1, which was seen to be associated with various H3K27ac-marked enhancers of limb genes which it activates during development.[28] A role for lineage-specific factors was proposed to allow the regulation of enhancers during development, and their ability to determine cell identity by providing control of basic gene expression in a tissue-specific manner.[2,29]

The binding of a particular factor to both enhancer and promoter, as does Pitx1, might also facilitate the physical interactions of these regions; we showed previously that Pitx1 both dimerizes and induces conformational change in the DNA upstream of the luteinizing hormone β-subunit gene promoter,[30] and other studies have also reported that interacting promoter-enhancer pairs contain common TF binding sites.[27] Notably, however, the *Cga* gene is expressed not only in the pituitary gonadotropes, but also in the thyrotropes, as it comprises part of the thyrotropin stimulating hormone, and also in the human blastocyst where it forms part of the human chorionic gonadotropin. In both cell types Pitx1 is not found, and in blastocysts transcriptional activation of this gene appears to utilize distinct regulatory elements,[14] suggesting the regulation by distinct factors and elements in the various cell types.

In the gonadotropes, hormonally-stimulated *Cga* gene expression likely also utilizes additional enhancer elements: even when knockdown of the eRNA had reduced *Cga* levels to ~5 % those in wildtype cells, levels of *Cga* mRNA were still elevated by the primary regulatory hormone, the hypothalamic gonadotropin releasing hormone (GnRH).[13] Moreover, chromatin conformation capture analysis indicated an additional region of interaction in the GnRH-treated cells.[13] Also in response to EGF stimulation, distinct classes of

stimulus-induced immediate early gene-associated enhancers were noted, and although the DNA looping in response to the EGF-treatment was abolished by depletion of the Integrator complex, the stimulus-independent looping was not affected, indicating distinct function for different enhancer elements.[18]

It is thus becoming increasingly clear that there are various classes of enhancers with distinct functions, only some of which determine cell-specific expression and cell lineage, while others may mediate the effects of specific stimuli. In line with this, the human genome was estimated to contain enough putative enhancers to allow a typical gene to be regulated by tens of different regulatory elements, which would allow enhancer activation in response to various stimuli in diverse tissues (reviewed by refs. 1,5). The levels of expression at the stimulus-responsive enhancers also change with the stimulus, such that the levels of mRNA and these eRNAs correlate,[6,7] and it was proposed that these alternative enhancers may be primed during development through the binding of pioneer transcription factors allowing their activation at later stages in development.[5]

This flexibility of potential usage of various genomic elements to enhance transcription of a particular gene in distinct settings is clearly advantageous as it increases the number of possible factors that might be utilized to activate it, and thus would allow gene expression in diverse cell types and developmental stages. A notable example is the *Tet1* gene, which is expressed abundantly in stem cells due to the actions of pluripotency factors, Oct4, and Sox2, that act through an enhancer located in the first intron.[33] As expression of these factors is reduced during differentiation, *Tet1* then utilizes a different TSS whose activity is not dependent on this enhancer, allowing expression of the same Tet1 protein from a transcript that differs at its 5'UTR.[31] However, that study noted that Tet1 in the brain appears not to be transcribed from either of these isoforms, and we have seen that in many fully-differentiated tissues, including the brain and pituitary gonadotropes, a different isoform is expressed that utilizes another distinct downstream TSS; the region upstream of one of TSSs described by Sohni et al.[31] acts as a transcriptional enhancer for expression of this isoform (Yosefzon and Melamed, unpublished).

The case of the Tet1 promoter and enhancer usage further corroborate previous observations that intragenic enhancers can function as alternative tissue-specific promoters in specific circumstances and that strong promoters in one tissue may act as enhancers in others.[26,32,33] Moreover, the varying utilization in distinct tissues of the genomic sequences upstream of the *Tet1* gene was corroborated by the presence of different histone modifications, and suggests these may indeed be related to the different behaviors of an enhancer to direct transcription of an eRNA as compared to a promoter which directs transcription of a spliced, polyadenylated transcript.

Perspective

Our findings, together with mounting evidence from other studies, suggest that eRNAs may comprise a pivotal element in enhancer function. Given that distinct enhancers, and thus also their eRNAs, appear to modify either basal levels of expression or increased rates of transcription in response to specific stimuli, they must be activated differently and likely employ various modes of action. However, the mechanisms involved in driving eRNA transcription, as well as their varied functions, are far from understood and may likely often be context-specific while some, grouped into "superenhancers," control multiple genes involved in cell type specification, through defining specific topologies in the genome.[34-36] The fact that so much of the genome now appears to have a regulatory function has placed new emphasis on understanding these mechanisms, which may form the basis of many diseases.[37,38]

Abbreviations

AR	androgen receptor
CDK8	cyclin-dependent kinase 8
Cga	glycoprotein hormones, α subunit
EGF	epidermal growth factor
ERα	estrogen receptor α
eRNA	enhancer RNA
GnRH	gonadotropin releasing hormone
H3K4me1	monomethylated histone H3 lysine 4
H3K4me3	trimethylated histone H3 lysine 4
H3K36me3	trimethylated histone H3 lysine 36
H3K27ac	acetylated histone H3 lysine 27
H3S10	histone H3 serine 10
HDAC	histone deacetylase
HOXA	homeobox A
HOTTIP	HOXA transcript at the distal tip
lncRNA	long non-coding RNA
MLL	mixed linage leukemia
NELF	negative elongation factor
PIC	pre-initiation complex
Pitx1	paired-like homeodomain1

RNAPII	RNA polymerase II
pS2-RNAPII	phosphorylated RNAPII serine 2
Tet	Ten-eleven-translocation 1
TF	transcription factor
TSS	transcriptional start site
UTR	untranslated region
WDR5	WD repeat domain 5

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