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Is Enhancer Function Driven by Protein–Protein Interactions? From Bacteria to Leukemia

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ABSTRACT

The precise regulation of the transcription of genes is essential for normal development and for the maintenance of life. Aberrant gene expression changes drive many human diseases. Despite this, we still do not completely understand how precise gene regulation is controlled in living systems. Enhancers are key regulatory elements that enable cells to specifically activate genes in response to environmental cues, or in a stage or tissue-specific manner. Any model of enhancer activity needs to answer two main questions: (1) how enhancers are able to identify and act on specific genes and (2) how enhancers influence transcription. To address these points, we first outline some of the basic principles that can be established from simpler prokaryotic systems, then discuss recent work on aberrant enhancer activity in leukemia. We argue that highly specific protein–protein interactions are a key driver of enhancer-promoter proximity, allowing enhancer-bound factors to directly act on RNA polymerase and activate transcription.

1 | Introduction

Transcription of DNA is a fundamental process of life. To respond appropriately to environmental cues and drive tissue specificity, organisms need to be able to turn genes on and off, and increase and decrease transcription appropriately. Transcription initiation requires the stable assembly of an RNA polymerase (RNAP) complex at a specific initiation site termed the promoter [1]. The length of the transcript is determined by elongation efficiency, along with termination signals, and the ultimate fate of the RNA product depends on its stability and nuclear export. What happens to RNA after it is transcribed is not the focus of this review; instead we consider what we know about mechanisms that allow for the precise control of gene transcription.

2 | Transcription Is Regulated by Enhancers

Enhancers were first identified in SV40 viral DNA, demonstrating an ability to increase transcription in cultured mammalian cells when paired with their cognate promoters [2]. Since then, our knowledge has expanded to recognize enhancers as a universal feature of gene regulation, essential for driving transcription in a highly developmental, tissue, and environment specific manner [3–5]. It is therefore not surprising that aberrant enhancer activity is increasingly being acknowledged as a driver of human disease [6–12].

Although enhancers are generally considered to be a eukaryotic innovation, enhancer-like elements also provide a crucial mechanism for gene regulation in prokaryotes [13]. Despite this

ubiquity, the true mechanistic activity of enhancers is still not fully understood. In this review, we discuss what is known about enhancer function, and relate it to our recent work on oncogenic enhancers in leukemia [10, 14]. From there, we explore what this tells us about enhancer function more generally.

3 | The Main Questions a Model for Enhancer Function Needs to Answer

Many different models of enhancer function have been proposed (comprehensively reviewed in [5]), but any model must explain two key questions. Firstly, how do enhancers identify and act on specific promoters? This question is underscored by observations that it is common for enhancers to bypass neighboring genes, with studies finding values ranging from 10% to 60% of enhancers that do not interact with the nearest promoter [15–18]. This argues that specificity is controlled by factors other than simple proximity along the linear sequence. A particularly elegant example of this is displayed by extensive work on the regulation of the alpha-globin locus [4], where the globin-specific enhancers are embedded in the nearby *NPRL3* gene, but guide erythrocyte specific expression of the globin genes without activating the *NPRL3* promoter [19].

Secondly, when an enhancer acts on a promoter, how does it influence transcription? It is worth considering the role of enhancer-like elements in bacteria to identify universal principles that apply across species.

4 | Bacteria: Basic Principles for Enhancer Function

Many of the principles of transcription were first established with experiments in bacteria [20, 21], including purification and structural analysis of the RNAP complex [22–24] and some of the early single molecule work studying transcription dynamics [25]. Initiation of transcription requires two key processes: stable binding of RNAP to DNA (i.e., the promoter), followed by melting of the DNA. A single strand is introduced into the active site, enabling dNTP binding and formation of the first phosphodiester bond. A subsequent transition to stable transcription is required to achieve productive elongation. At high-affinity promoter elements, RNAP binding can be achieved without additional factors. However, where promoter sequences are suboptimal, additional regulatory elements (e.g., enhancer-like elements) are required to enable stable RNAP binding and promoter melting. Suboptimal promoters are not simply a problem for the cell to overcome but represent an opportunity to introduce layers of modulation to the transcription of a gene. Control of the factors present at enhancers therefore provides a mechanism to regulate gene activation, for example, making transcription responsive to the environment or other specific cues.

In bacteria, anchoring RNAP to the promoter is achieved by a set of DNA-binding proteins called sigma (σ) factors [26]. Canonical σ^{70} factors direct RNAP binding to −35 and −10 promoter elements, and facilitate DNA melting. In contrast, the σ^{54} family, which recognizes distinct promoter sequences, lacks this unwinding activity (Figure 1A). Its ability to isomerize and activate transcription is entirely dependent on accessory factors, known as bacterial enhancer-binding proteins (bEBPs) [27, 28]. bEBPs bind to sequences typically 100–150 bp away from the promoter [28] but can function up to 3 kb away [29], and actively promote the formation of loops between the enhancer-like element and RNAP-bound promoter, directly binding to σ^{54} to induce DNA melting [30] (Figure 1B). In this way, bEBP-binding sites act analogously to eukaryotic enhancers. Looping is promoted by the integration host factor (IHF), helping bend DNA to bring bEBPs into the vicinity of the promoter [27, 31]. These loops disappear when transcription is initiated [30], suggesting they are a temporary occurrence needed only for the early stages of transcription (Figure 1C).

The observation that transcription disrupts loop formation fits with the specific role for bEBPs in σ^{54} regulation. Although σ^{54} binding stabilizes RNAP binding, it also blocks DNA loading into the cleft of RNAP, keeping the complex in an inhibited state [28]. Interaction with bEBPs drives conformational changes, further DNA melting, loading of the DNA into the cleft, stabilization of the transcription bubble, and ultimately productive elongation [28]. Once productive elongation begins, bEBP contact with the σ^{54}:RNAP complex is no longer necessary and may even be inhibitory. Thus, these enhancer-promoter loops are transient, a point we will return to.

Recent 3C work has revealed higher order DNA looping structures in bacteria [32], similar in appearance to higher order structures in eukaryotic cells (discussed in section 5). Importantly, these bacterial DNA loops are driven by transcription [32], likely due to protein–protein interactions such as bEBP:σ^{54}:RNAP, but could also be driven by the interaction of transcription complexes themselves.

5 | General Features of Eukaryotic Enhancer Function

Many aspects of transcriptional regulation in eukaryotes are similar to bacteria, but the protein complexes are bigger, the genomes are larger and contain many more genes, and the DNA has an additional regulatory layer created by the histone protein–DNA complex termed chromatin [33–35]. In multicellular organisms, more intricate control of gene expression is also crucial, adding complexity to the regulatory mechanisms required. As a key tool for driving higher levels of transcription from the promoter, enhancers are crucial regulatory elements, separated from their target genes by much longer distances than in bacteria [3, 5, 28].

In general, eukaryotic enhancers function as binding sites for sequence-specific DNA binding proteins, known as transcription factors (TFs). Active enhancers are associated with nucleosome-free or "open" regions (likely because they are bound by TFs), flanked by high levels of histone acetylation (especially H3 lysine-27, H3K27ac) as well as an enrichment for H3 lysine-4 monomethylation (H3K4me1) [36–39]. They are actively transcribed, typically producing short, unstable RNAs termed eRNAs [1] that are often rapidly degraded [40]. However, some enhancers are associated with the transcription of more stable long noncoding RNAs (lncRNAs) [41]. Enhancers can exist in large clusters termed super-enhancers that are often associated with cell

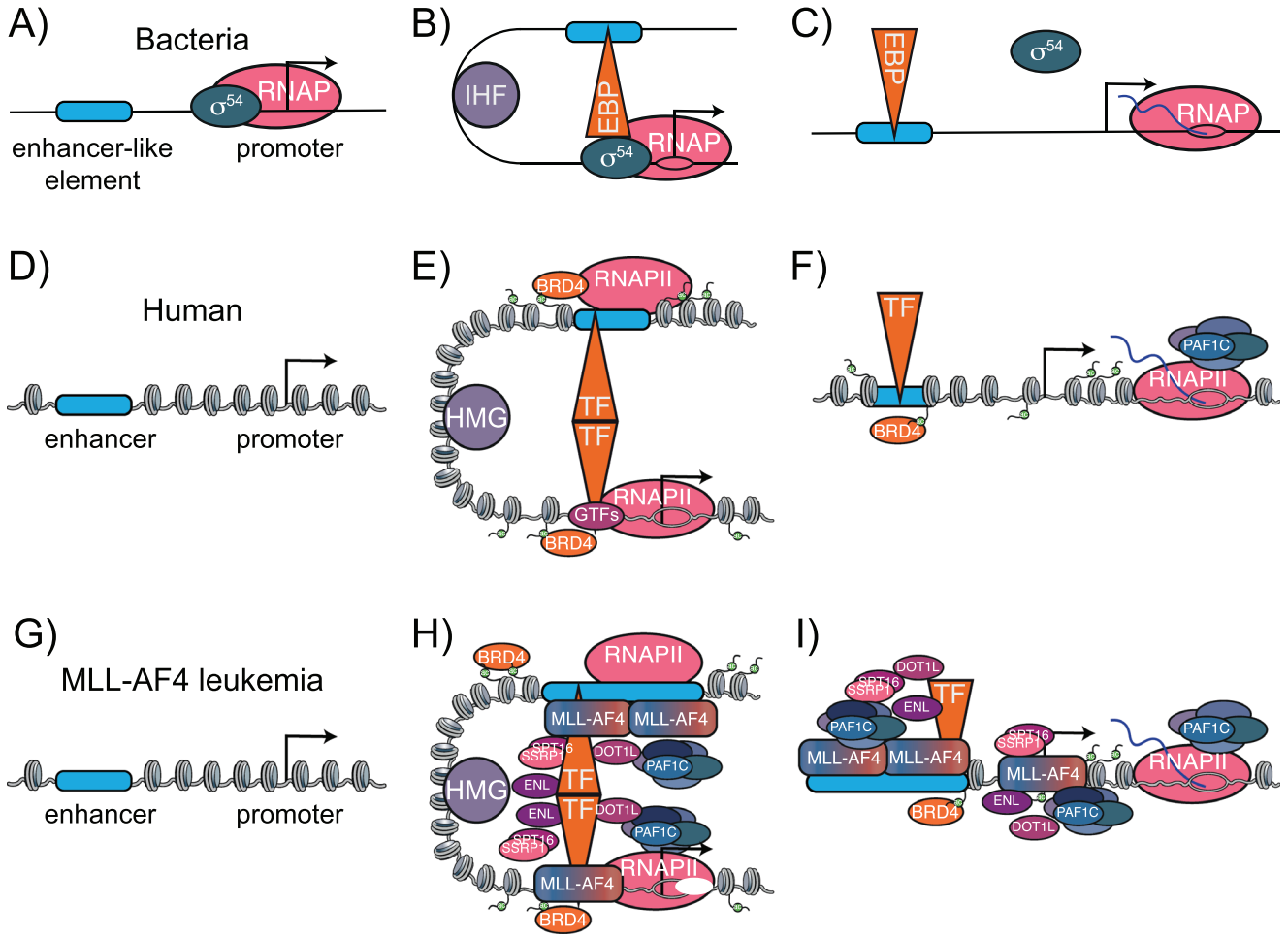

FIGURE 1 | Bacterial enhancers reveal principles of mammalian enhancer function. (A) σ^{54} anchors RNA polymerase (RNAP) to the promoter. (B) Bacterial enhancer-binding proteins (EBP) bind to an upstream enhancer-like element and directly contact the σ^{54}:RNAP complex, causing a conformational change and aiding in the formation of the transcription bubble at the promoter. Integration host factor (IHF) proteins help bend the DNA to make this loop structure possible. (C) Productive transcription elongation initiates and the loop collapses. (D) In human cells, an analogous process occurs, except that the DNA is complexed within nucleosomes, the sequence distance between enhancer and promoter is much larger (not to scale), and RNA polymerase II (RNAPII) binds to the enhancer as well as the promoter. (E) Once a gene becomes activated, nucleosomes are moved to allow for the binding of transcription factors (TFs), the promoter and enhancer become acetylated, and the RNAPII preinitiation complex is assembled at the promoter. A transient loop is formed by protein–protein interactions, potentially between TFs bound at either locus that then directly interact with the RNAPII complex, helping stabilize complex formation and DNA melting. Similar to IHF, HMG proteins may help bend the DNA, but it is likely that cohesin-mediated loop extrusion also aids in loop formation (not shown). Factors such as BRD4 are important for downstream aspects of transcription activation but do not aid in loop formation. (F) The loop again collapses as productive transcription elongation occurs. (G) In Mixed Lineage Leukemia (MLL)-AF4 leukemias, the enhancer–promoter setup begins the same. (H) Binding of MLL-AF4 and the associated transcription elongation machinery creates a large, highly transcriptionally active complex at both the enhancer and promoter. MLL-AF4 enhancers spread over tens of kb. Crosstalk between MLL-AF4 complex components at the enhancer and promoter facilitates loop formation (likely aided by TFs as well as HMG proteins and cohesin, as described above). Upon induction of productive elongation, the loop collapses but MLL-AF4 complex components are able to rebind and start the process, maintaining high levels of transcription.

identify or disease states [42–44]. Whether the super-enhancer concept is a useful model for enhancer function has been the topic of some debate [45], but they are generally recognized to be regions of dense protein binding and activity, and are often visually identifiable.

Physical proximity between an enhancer and its target promoter, sometimes over very long distances, is a common feature of active enhancers. Although there may be examples where proximity does not appear to be important [46, 47], it is generally accepted to be a prerequisite for gene activation [3, 15, 18, 48–50] (Figure 1D–F). In eukaryotes, large looping domains of chromatin, bounded by CTCF, are thought to be created through the loop extrusion activity of cohesin [51, 52], termed topologically associated domains (TADs) [53]. However, recent work in yeast has found that loop extrusion-deficient cohesin mutants retain the ability to entrap chromatin and form loops, especially TADs, arguing that extrinsic processes such as transcription may instead drive looping [54]. Within TADs, enhancers and promoters can interact, sometimes as a hub or cluster [55], through as yet unresolved mechanisms. One consequence of TADs is the insulation of enhancers from interacting with promoters outside

the TAD [4, 5, 11, 56], providing a mechanism to restrict potential targets for gene activation. Loop formation is likely to be the sum of a complex series of interactions; in this review, we focus specifically on enhancer–promoter interactions and do not address the formation of larger domains such as TADs.

6 | Controversies

The universality of histone acetylation at both active promoters and enhancers has been recognized [38, 39], and although no single acetylation mark is likely to be essential [57], acetylation is generally thought to function by a combination of direct chromatin relaxation and providing a binding site for chromatin proteins [58, 59]. However, one intriguing model has proposed that histone acetylation is simply a by-product of acetyltransferase localization. In this model, enhancers are platforms to enrich for acetyltransferase activity, creating a concentration of acetylated TFs that diffuse toward the promoter without the need for direct enhancer–promoter contact [60]. Although an interesting idea, it remains untested. It seems likely that the fundamental features of enhancer function should be conserved in organisms without chromatin, such as the bacterial enhancer-like elements discussed above.

The role of liquid–liquid phase separation in transcription is hotly contested and has been extensively reviewed elsewhere [11, 61, 62]. Many of the proteins found at enhancers, including TFs and the coactivator complex Mediator, contain intrinsically disordered regions, which can cluster in droplets in vitro [63, 64]. Assembly of a high concentration of these factors in vivo, particularly at super-enhancers, is proposed to drive a similar assembly in the nucleus, promoting gene activation at the promoter [63, 64]. Although there is some evidence for the ability of condensates to generate mechanical force, bringing DNA molecules together in vitro [65], in vivo experiments argue that phase-separated condensates do not drive enhancer–promoter physical proximity [66, 67]. However, phase separation may promote functional interaction between enhancer-bound proteins and the preinitiation complex at the promoter. As discussed in a recent review [5], it is difficult to explain how condensate formation can mediate enhancer–promoter selectivity, especially where there is extensive crowding of transcriptional units and the enhancers that regulate them.

As already mentioned above, when enhancers become active, they generally come within close proximity of the promoter, an effect that has been measured in cells using both imaging and chromosome conformation capture (3C) technologies [5]. Recent exhaustive studies of enhancer function across different developmental stages in mice and *Drosophila* have emphasized the strong correlation between enhancer–promoter proximity and gene activation [18, 50]. However, a more precise temporal link between enhancer–promoter convergence and gene activation has not been conclusively established, with some work suggesting that enhancer–promoter proximity is not a good predictor of active transcriptional bursts [67, 68]. In the early stages of *Drosophila* development, enhancer–promoter proximity and gene activation is more ambiguous [50]. Further, at specific loci, proximity may not be necessary for enhancer function at all [46, 47]. Although some of these disparities can be explained by technical differences between imaging and 3C techniques [49], some models for enhancer function propose that proximity is not functionally significant (discussed in [5]). Consideration of the conserved principles of enhancer function could explain why proximity is likely necessary, at least at specific stages of transcription initiation.

7 | A Basic Model for Enhancer Function

Although many aspects of bacterial gene regulation differ to eukaryotes, especially multicellular organisms (not least of which the addition of chromatin adds important, additional complexity [35]), several essential principles are maintained. The binding of RNAP (RNA polymerase II [RNAPII] in eukaryotes) at a gene promoter is dependent on specific protein-DNA and protein–protein contacts mediated by general transcription factors (GTFs), together forming the preinitiation complex, driving conformational changes, DNA melting, engagement, and ultimately productive elongation (the specific details are reviewed elsewhere [1, 35]). By limiting the affinity of individual factors for the promoter, multiple components must be recruited, providing additional levels of regulatory control. As in bacteria, the ability to loop DNA allows these recruitment sites to be located away from the promoter itself, that is, at enhancers (Figure 1D–F). Since sequence proximity is not a limitation for distal enhancers, it is possible for a gene to be regulated by multiple sequence elements, permitting activation under different environmental or developmental conditions, thus increasing complexity in regulation.

What, then, is the role of the enhancer in gene activation? Based on the bacterial model, we propose that enhancers function to facilitate the assembly and activation of the initiating RNAP at the promoter. Current high-resolution 3C methods such as Region Capture Micro-C (RCMC) or Micro-Capture-C (MCC) have made the striking observation that enhancer–promoter interactions are precisely localized to TF binding sites, and therefore likely driven by specific protein binding patterns [69–71]. Given the dynamic binding of TFs [25, 72], this fits with the idea that these loops might be transient. The snapshot nature of 3C techniques, averaged over a population of cells, can give the misleading impression that loop formation is stable. However, single-cell super-resolution imaging approaches have shown that individual cells display unique interaction patterns [73, 74]. Thus, TAD patterns from cell populations are best described as "statistical properties of the genome" (as recently described in [49]) rather than being discrete or stable structures. Live imaging of cohesin-dependent loop formation also shows that such loops occur rarely and are maintained for only 5–20 min at a time, indicating a high level of dynamism [51, 52]. This is still consistent with 3C data, as HiC can identify loops even when they only occur in 2–4% of the cells at any given moment [75]. Together, these results are best explained by stochastic and dynamic loop formation in individual cells, and RCMC and MCC tracks are compatible with the concept of dynamic TF driven protein–protein interactions that are constantly collapsing and reforming. It is worth noting, however, that this dynamic behavior may not explain all loci, as it is possible that at individual sites, high levels of combinatorial activity may produce relatively stable interactions. As the tools to study enhancer behavior develop, we may find that distinct sites display such stable structures.

If the main purpose of the enhancer is to recruit factors that transiently stabilize and activate RNAP, enhancer–promoter proximity is likely a prerequisite of gene activation, representing the need for specific protein–protein contacts. Some evidence comes from live cell imaging of enhancer proximity at the *Sox2* locus [46, 67]. The *Sox2* enhancer was found to be essential for the formation of an RNAPII phase-separated condensate associated with transcription, but importantly, transcriptional bursting strongly correlated with proximity to the condensate rather than the enhancer [67]. This indicates a requirement for the enhancer to form a functional RNAPII complex, potentially mirroring what is observed in bacteria (Figure 1). These results also fit with the collapse of the loop upon transcription initiation, which may explain why the transcriptional burst does not correlate with enhancer–promoter proximity [67]. Interestingly, as in bacteria [32], transcription alone contributes to looping structures in eukaryotes [76–78].

However appealing it might be to think that TFs themselves drive enhancer–promoter interactions [70], the identity of these factors, and the mechanisms by which they achieve this, remain unclear. In the next section, we explore what proteins might be driving these contacts in mammalian systems, focusing on a rare leukemia that is considered to be primarily a disease of aberrant transcription.

8 | MLL-AF4 Driven Leukemias and Transcription—Not Just About Promoters

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer [79, 80], while acute myeloid leukemia (AML) affects ~3000 people/year in the UK, mostly over the age of 60 [81]. Although these diseases may not seem to have much in common, both can be driven by translocations of the same gene, *Mixed Lineage Leukemia* (*MLL*, also known as *KMT2A*), where they are generally responsible for a poor prognosis [80, 82, 83]. Most *MLL* rearrangements (*MLL*r) fuse MLL in-frame with different partners that create fusion proteins, the most common of which is MLL-AF4 [84]. These fusions retain the DNA binding domains of MLL, meaning that MLL fusion proteins bind to genes and aberrantly activate them, mainly through the recruitment of a large transcription elongation complex [85–88]. The classic model posits that *MLL*r leukemias are a transcriptional disease caused by MLL fusion protein binding to oncogene promoters, where they aberrantly drive transcription elongation [86–88].

Work from us has shown that this model is over-simplistic, with a role for MLL-AF4 in enhancer activation. The MLL-AF4 complex binds to enhancer sequences as well as promoters, driving enhancer–promoter proximity [10]. We also identified a new component of MLL fusion protein complexes, the FAcilitates Chromatin Transcription (FACT) complex [10]. FACT is an H2A/H2B chaperone, originally implicated in transcription elongation [89], recently also implicated in maintaining nucleosome stability at the promoter and across the gene body [90]. Considering that MLL fusion protein complex components normally function by promoting transcription elongation, how do they activate enhancers?

9 | What Can MLL-AF4 Tell Us About Enhancer Function?

MLL-AF4-bound onco-enhancers are particularly active, with MLL-AF4 driving broad domains of open chromatin and a high density of enhancer–promoter contacts [10]. MLL-AF4 assembles the same complex of components at enhancers and promoters, including ENL, DOT1L, PAF1, and FACT (Figure 1G–I). We suggested that these unusually large enhancers, and enhancer–promoter contact, are driven by multivalent interactions of the MLL-AF4 complex and high levels of transcriptional activity, as evidenced by elevated eRNA transcription [10].

Given that MLL-AF4 is known to upregulate transcription, one explanation may be that these highly active enhancers are simply a by-product of this process. That is, enhancer–promoter proximity could simply be a consequence of crosstalk between transcription complexes at enhancers and promoters. Indeed, transcription has been demonstrated to drive 3D structures in some cases. Using an auxin degron system, Zhang et al. found that RNAPII is essential for maintaining many enhancer–promoter interactions within TADs [77]. Similarly, using an elegant inducible *GAL7/10/1* model, Chapard et al. found that induction of transcription creates a boundary that has local connections with nearby active promoters [78]. Upon degradation of the cohesin component Smc3, these contacts were conserved, suggesting they are due to transcription [78]. This fits with the idea of specific activators interacting with specific promoter complexes, like the bEBP:σ^{54} interaction, as well as looping observed between active genes in bacteria [32].

If it is simply transcription that induces enhancer–promoter contacts, disruption of transcription should also disrupt enhancer–promoter proximity. However, we found that inhibition or degradation of the co-activator BRD4 disrupts eRNA transcription (especially at super-enhancers, Figure 2) and gene transcription, but has no impact on enhancer–promoter proximity [66]. This implicates BRD4 in promoting transcription, but not the protein–protein interactions that likely drive enhancer–promoter contacts.

Together, these findings indicate that it is not transcription per se that drives proximity, and different factors can have cellular context-dependent functions. Thus, BRD4 and the Mediator complex function in promoting later stages of transcription initiation and elongation such as promoter melting and pause release [1]. Such regulatory events are essential for transcription, but downstream from establishing the preinitiation complex, which may be the key event mediated by looping and enhancer–promoter proximity. Before we bring these observations together into a more general model for enhancer function, it is worth considering another important aspect of enhancers—why are they located far away from promoters?

10 | Why Do Enhancers Act at Distance?

If enhancers function mainly to increase preinitiation complex formation, what evolutionary advantage is there to having enhancers so far away? One explanation is that it creates the

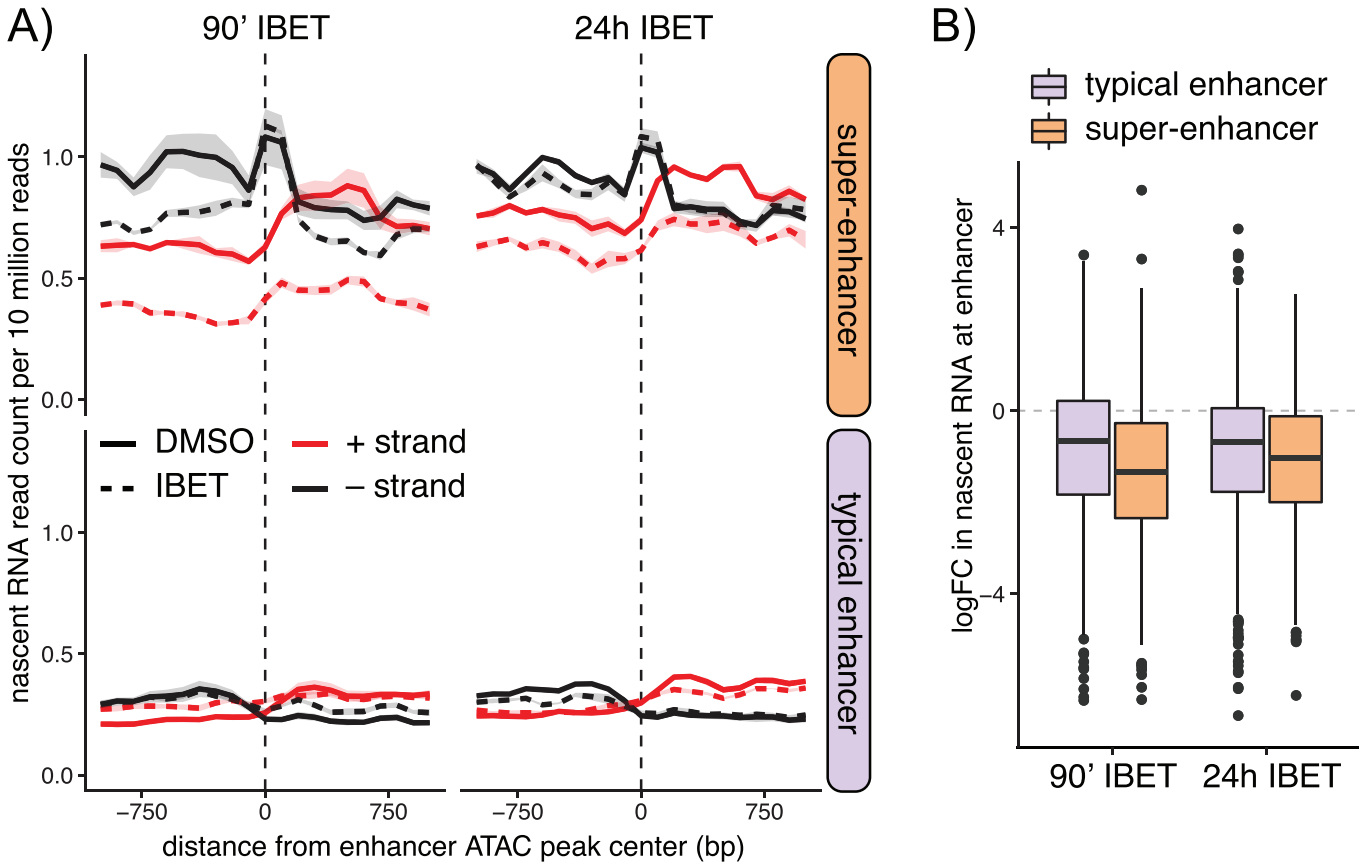

FIGURE 2 | Disruption of enhancer RNA transcription following BET inhibition. (A) Nascent RNA-seq at enhancer ATAC peaks in the MLL-AF4 leukemia SEM cell line, following treatment with DMSO or 1 μM IBET-151 for 90 min or 24 h. (B) Log2-fold change in nascent RNA transcription at enhancers following IBET-151 treatment. Data reanalyzed from [66].

opportunity for multiple mechanisms to activate the same gene—different enhancers can be activated in different contexts to independently regulate a single target. Designing an equivalent system at the promoter would provide less freedom for multiple regulatory elements to be available.

Interestingly, the ability of an enhancer to activate transcription is not independent of enhancer–promoter distance. Recent studies have demonstrated that more distal enhancers show reduced activation potential, indicating that the mechanisms that drive gene activation are sensitive to sequence separation [91–93]. This suggests the intriguing possibility that, along with the sequence of the enhancer itself, sequence proximity to the promoter provides an additional mechanism to modulate gene activation. The combinatorial action of enhancers also appears to be sensitive to promoter distance. Integration of a weak enhancer between a promoter and strong enhancer can act synergistically, potentially by bridging the gap between the two [93]. Whether enhancer–promoter interaction frequency is also increased in these combinatorial models remains an open question.

A requirement for distal enhancers may provide a difficulty barrier, discouraging promiscuous gene activation. 3C studies indicate that bacterial genomes have "structure" that seems to be driven by gene activation [32]. This makes sense if we consider that these protein complexes have affinity for each other, and when bound to DNA they are likely to interact just through random diffusion, thus creating proximity (which may not be functional but simply reflect interaction dynamics). For random interactions to drive a transcriptional burst, the concentration of complexes would need to reach a certain threshold to make productive interactions with the promoter. Thus, this may be a way to select for persistent activation signals, decreasing spurious transcription signals emanating from a noisy background.

Maintaining 3D interactions appears to be actively challenged by dynamic processes regulating chromatin structure. Repression complexes have a propensity to form clusters, as shown by long-range interactions of PRC complexes [75]. Cohesin-mediated looping has been proposed to function in part to "stir up" the cell, breaking these interactions and requiring them to reform [75]. This antagonizes stable interactions, preventing stasis where all active regions cluster together and repressed regions cluster together. Indeed, cohesin loss results in long-range active gene interactions [76, 78]. This model requires a continuous process of enhancer–promoter complex formation to maintain gene expression, driving specificity and selection. This is not incompatible with TADs helping to limit external interactions and increase the opportunity for internal interactions.

Thus, proximity is a reflection of protein–protein interactions. If you disrupt the enhancer complex you should expect proximity to decrease—so why are some factors essential for proximity but others not?

11 | What Is Happening at Enhancers in Normal Cells?

It remains to be seen whether components of the MLL-AF4 complex are essential for enhancer function in other contexts. The evidence so far suggests their impact may be more subtle. PAF1 is essential for enhancer–promoter interactions in *MLL*r leukemia but only appears to have a mild impact on enhancer activity in multiple myeloma [10]. However, its role in transcriptional elongation is maintained. Similarly, FACT is essential for enhancer activity in MLL-AF4 leukemias [10], but is apparently dispensable for enhancer–promoter interactions in K562 cells [90]. Thus, the roles for PAF1 and FACT in 3D genome structure that we observed may be a noncanonical role induced by the MLL-AF4 fusion protein. There is some overlap between MLL-AF4 enhancers and super-enhancers, so it is possible that MLL-AF4 generates a highly active subclass of super-enhancer [10].

Recent work indicates that enhancers are important for phase-separated condensate formation, at least at the *Sox2* super-enhancer [67]. This may not hold for other enhancers, but it raises the possibility that enhancers are required to act as a platform for the localized concentration of factors for RNAPII stabilization and assembly. In the case of MLL-AF4 leukemias, genes are particularly highly transcribed, suggesting rapid rates of forming and reforming transcription-competent complexes. This may generate a greater need for specific factors such as PAF1 and FACT. Given the multivalent interactions underpinning this complex [10, 94–98], we believe that it serves as an extreme example of protein–protein interactions driving enhancer–promoter proximity. These interactions result in the spreading of MLL-AF4 along chromatin into long enhancer domains but also stabilize binding of MLL-AF4 complexes at the promoter, producing a high density of enhancer–promoter interactions (Figure 1H). The importance of the multivalent interactions is highlighted by the fact that the loss of one of several complex components (FACT, PAF1, H3K79 methylation, or MLL-AF4 itself) is sufficient to collapse these enhancer–promoter contacts [10, 99].

In a more normal context, however, eukaryotic enhancer behavior is likely more comparable to the bacterial system (Figure 1). Both involve factors that bend DNA (IHF/HMG proteins), and factors that bind to enhancer sequences, interacting directly with the RNAP complex (bEBP/TFs) to anchor it to DNA and aid with preinitiation complex assembly and isomerization. Interestingly, Larke et al. found that most genes are regulated at the level of initiation [100], and that the alpha-globin and beta-globin enhancers function primarily through transcription initiation [100]. This suggests that initiation could be a common step regulated by most enhancers, which fits with the bacterial model.

A role for protein–protein interactions driving enhancer–promoter contact has been convincingly demonstrated for the TF LDB1. Tethering LDB1 at the silent globin gene promoter leads to its activation, inducing interaction with the locus control region [101–103], and degradation of LDB1 disrupts contact between LDB1-bound promoters and enhancers [104]. LDB1 is likely one of several factors that act in this way. Not all proteins found at the enhancer necessarily directly contribute to enhancer–promoter contacts or stabilization of RNAP initiation. Coactivators that act downstream from this, which directly promote transcription itself, are likely not involved in loop formation. This could also include coactivators such as BRD4 and Mediator—as well as PAF1 and FACT. Conversely, other factors may help promote enhancer–promoter proximity without directly impacting RNAP stabilization or initiation, such as the central role of CTCF and cohesin in bringing together the *MYC* gene and its super-enhancer, almost 2 Mb away [48, 66].

This model implies that the compatibility of TFs bound at the enhancer and promoter may determine whether the two colocalize, and hence the ability of the enhancer to activate the gene. High-throughput reporter assays in *Drosophila* and mouse have identified widespread differences in the ability of enhancer elements to activate different promoters [105, 106], with more than half of the murine enhancers tested showing significant preferences [106]. In contrast, a similar study in human K562 cells found only a mild specificity of enhancers for promoters [107]. Importantly, since these assays involve constructs with the enhancer and promoter separated by only a short sequence, reporter gene activation is independent of chromatin looping. The results are therefore a better reflection of later stages in enhancer activity, i.e. the ability of enhancer-bound factors to activate target genes, downstream of enhancer–promoter contact.

Ultimately, there is much that we still do not understand about the drivers of enhancer–promoter proximity. Our model of protein-driven contact is unlikely to be convincingly demonstrated until the proteins responsible are identified. Ultra-high-resolution 3C technologies such as MCC, identifying the foci of enhancer–promoter contacts, combined with acute protein degradation of the factors bound there, may provide effective approaches to dissect the drivers of interactions for specific genes.

12 | Enhancers as Therapeutic Targets in Human Disease

The importance of enhancers in disease has been highlighted by therapeutic strategies targeting them to perturb the transcription of key genes. Recently, a CRISPR/Cas9-mediated cure for thalassemia was demonstrated, where directed mutations in the enhancer for *BCL11A* reduce its expression in erythropoiesis [108], disrupting repression of fetal globin and alleviating disease symptoms. This approach has been successful in the clinic, curing people of a disease that otherwise requires a lifetime of transfusions [108].

Enhancer editing may also work for cancer, but the approach is more difficult here. The advantage of thalassemia is that not all cells need to be successfully targeted to treat the disease, whereas if even a few cancer cells escape editing the patient will relapse following clonal expansion.

Instead of targeting oncogenic enhancers directly, work has focused on proteins that drive enhancer function. To some extent, this was part of the rationale for BET inhibitors, blocking the acetyllysine-binding bromodomain of BRD4 [109–111]. Since BRD4 binds at the *MYC* super-enhancer, and targeting BRD4 reduces *MYC* expression, it could be used to indirectly target *MYC*-addicted cancers [110]. There have been two problems with

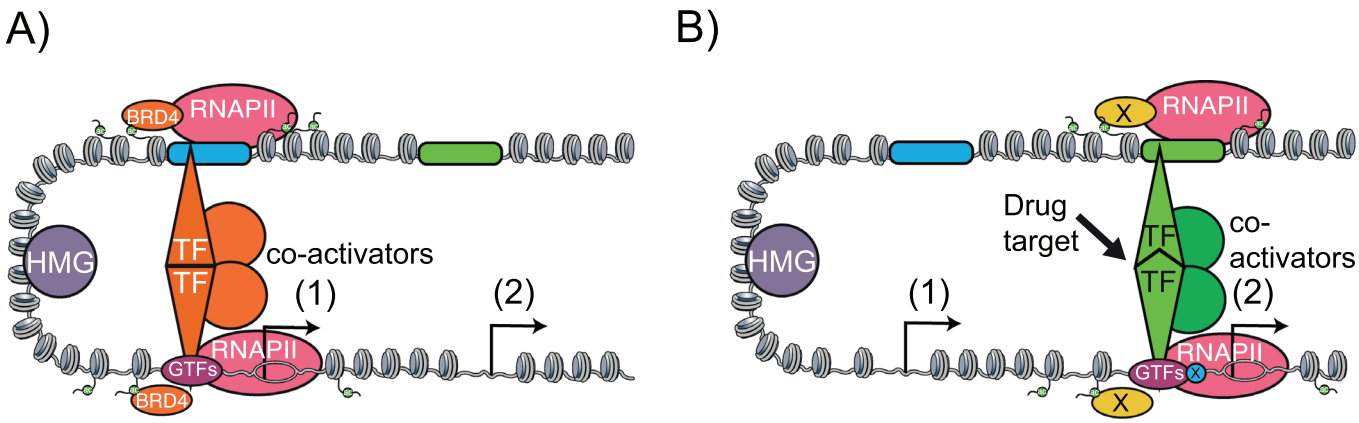

FIGURE 3 | Unique protein–protein interactions may drive enhancer selectivity. (A) Compatibility of interacting transcription factors (TFs) and the coactivators they recruit bound at the blue enhancer and promoter (1), allow the enhancer to specifically activate promoter (1) but not promoter (2). (B) When a different TF/coactivator complex is bound at the green enhancer, they are instead competent to assemble the protein complex bound at the promoter (2), which may consist of alternate coactivators at the promoter (tan oval X) and additional components of the GTF complex (blue circle X). A drug targeting the green bridging TF interaction surface would specifically inhibit outputs from the green enhancer, allowing targeted disruption of specific gene expression.

this approach. Firstly, it is not clear exactly what BRD4 does at enhancers, and its main function may not be enhancer-directed at all [66]. Secondly, the lack of specificity for target genes may drive toxicity issues [112].

However, in cancers in which enhancer activity is known to be critical, targeting enhancers may be sufficient to produce an antioncogenic effect. Multiple myeloma is associated with widespread activation of onco-enhancers, alongside a dependence on the endogenous immunoglobulin heavy chain enhancer in many cases [113–115]. Myeloma cell lines are particularly susceptible to inactivation of the H3K27 acetyltransferases P300/CBP, indicating a key role for these enhancer-associated factors [116, 117]. A small molecule inhibitor targeting the P300/CBP bromodomain is currently showing promise in clinical trials for multiple myeloma and other malignancies [117].

The key to targeting enhancers will be to better understand how different classes of enhancer–promoter pairs function, and what specific protein–protein interactions drive them. With this information, it may be possible to achieve specificity in targeting key genes (Figure 3).

13 | Conclusion

Enhancers are bound by specific factors, which directly interact with the RNAPII machinery to promote preinitiation complex formation and productive elongation. Unique protein–protein interactions may drive enhancer–promoter specificity, with specific coactivators in crosstalk with specific promoter complexes (Figure 3 and [1, 118]). This could be driven by TFs and coactivator interactions, or by direct TF interactions (Figure 3), depending on the enhancer–promoter context. Thus, each enhancer acts on the particular promoter(s) through the compatibility of protein complexes bound at each site, as in bacteria (Figures 1 and 3). Following promoter escape, these interactions are disrupted and the loop collapses (Figure 1). Loop formation is aided by factors that bend DNA (Figure 1), and cohesin-mediated domain formation that reduces the search space [119]. This basic model is compatible with the existence of higher order chromatin structures that either isolate or enable specific enhancer–promoter interactions [4, 5, 56, 120].

MLL-AF4 leukemia subverts this system by taking advantage of the fact that enhancers already display low-level RNAPII recruitment and transcription [1]. MLL-AF4 bound at enhancers concentrates its associated complex of elongation factors, amplifying eRNA transcription [10]. Cobinding of the MLL-AF4 complex at promoters and enhancers creates structural and functional compatibility, driving proximity via protein–protein interactions (Figure 1). This generates a powerful transcription-activating feedback loop and is likely a major driving force for the MLL-AF4 complex in causing leukemia, also contributing to heterogeneity among patients [10, 14].

Author Contributions

Thomas A. Milne and Nicholas T. Crump: writing–original draft, editing and creating figures, funding acquisition. **Nicholas T. Crump:** analysis of data.

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Conflicts of Interest

Thomas A. Milne and Nicholas T. Crump are both shareholders in and consultants for Dark Blue Therapeutics.

Data Availability Statement

BET inhibitor data reanalyzed from [66] and available from the Gene Expression Omnibus (GEO) with the accession code GSE139437.

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