Phase Separation and Transcription Regulation: Are Super-Enhancers and Locus Control Regions Primary Sites of Transcription Complex Assembly?

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It is proposed that the multiple enhancer elements associated with locus control regions and super-enhancers recruit RNA polymerase II and efficiently assemble elongation competent transcription complexes that are transferred to target genes by transcription termination and transient looping mechanisms. It is well established that transcription complexes are recruited not only to promoters but also to enhancers, where they generate enhancer RNAs. Transcription at enhancers is unstable and frequently aborted. Furthermore, the Integrator and WD-domain containing protein 82 mediate transcription termination at enhancers. Abortion and termination of transcription at the multiple enhancers of locus control regions and super-enhancers provide a large pool of elongation competent transcription complexes. These are efficiently captured by strong basal promoter elements at target genes during transient looping interactions.

1. Introduction

RNA polymerase II (Pol II) is efficiently recruited to individual enhancer elements of locus control regions (LCRs) and super-enhancers (SEs), and initiates bi-directional transcription. The high concentration of transcription factors, Mediator, and other co-activators at LCRs and SEs establish an environment for the efficient assembly of transcription complexes. The essence of our hypothesis is that transcription termination and abortion releases elongation competent transcription complexes from LCRs and SEs, which are then transferred to high-affinity basal promoters at target genes (Figure 1). Important aspects of this hypothesis are introduced in the following sections, and we end this essay with a more detailed description of the Pol II transfer model using the human β-globin gene locus as an example.

1.1. Enhancers and Promoters Recruit Transcription Complexes and Initiate Transcription

Promoters are defined as DNA elements that recruit transcription complexes for the synthesis of coding and non-coding RNA.[1,2] Enhancers are defined as DNA elements that positively regulate transcription at promoters over long distances in a position- and orientation-independent manner.[1,2] However, high-throughput RNA- and ChIP-sequencing studies revealed that many enhancers recruit Pol II and initiate transcription of enhancer RNA (eRNA), thus blurring the functional distinction between enhancers and promoters.[3–6] Enhancers and promoters contain multiple binding sites for transcription factors that are either ubiquitously expressed or restricted in expression to specific cell types, developmental stages, or other stimuli.[7] The transcription factors bind to promoters and enhancers and establish regions of open chromatin, which are detected as heightened sensitivity to DNase I and referred to as hypersensitive sites (HSs).[8] Enhancer and promoter associated HSs are nucleosome-free or are associated with unstable nucleosomes containing histone variants.[1,2,7–9] Nucleosomes flanking enhancers are characterized by the presence of specific histone marks, including mono-methylation at H3K4 (H3K4me) and acetylation at H3K27 (H3K27ac).[9] The nucleosomes at active promoters contain elevated levels of tri-methylated H3K4 (H3K4me3), which are also found at highly transcribed enhancers.[10]

Transcription at most, but not all, enhancers proceeds in both directions.[3,11,12] Bi-directional transcription also occurs at promoters but it is not as common as at enhancers.[13] Several new studies point to differences in promoter and enhancer associated transcription. Enhancer transcription produces relatively short noncoding RNA and is terminated by Integrator and/or the WD-domain containing protein 82 (WDR82), a component of the Set1A/Set1B histone H3K4 methyltransferase complex that is recruited to
transcription start sites (TSSs) by phosphorylated Pol II.\(^\text{14,15}\) Furthermore, transcription at enhancers is unstable and often leads to abortion of elongation.\(^\text{16,17}\) In contrast, transcription initiation at most Pol II promoters is stable and produces long mRNAs. These are interesting observations indicating that the mechanisms conferring transcription initiation, elongation, and termination are different at enhancers and promoters.

Topological studies revealed that enhancers come in close proximity to target gene promoters during transcription activation.\(^\text{18–20}\) In fact, forced looping between an enhancer and a promoter led to transcription activation.\(^\text{21}\) According to current gene activation models, the Mediator complex, a transcriptional coactivator, forms a physical bridge between distant regulatory regions and promoters, thereby promoting looping.\(^\text{22}\) Transcription of at least a subset of genes regulated by enhancers occurs in bursts indicating a discontinuous process of transcription complex recruitment, assembly, and/or conversion to elongation competent forms.\(^\text{23–25}\) The bursting phenomenon suggests that enhancer/promoter contacts may be transient and infrequent. Indeed, recent high resolution imaging experiments provide evidence for transient encounters between enhancers and promoters.\(^\text{26}\) The duration of these contacts may vary depending on the number of interacting molecules.

**Figure 1.** RNA polymerase II transfer model. Depicted are steps involved in the recruitment of Pol II to SEs, assembly into elongation competent transcription complexes, transcription initiation and elongation, abortion and termination, and transfer to target genes. Transcription factors recruit Mediator and other co-regulators to SEs. Mediator recruits Pol II and assembles a fraction into elongation competent transcription complexes. Transcription is initiated by phosphorylation of the CTD. Early abortion and transcription termination conferred by Integrator releases Pol II, which is dephosphorylated and transferred to target gene promoters.

1.2. LCRs and SEs Are Centers of Efficient Pol II Recruitment and Transcription Complex Assembly

LCRs are powerful, composite DNA-regulatory elements containing several HSs that function together to mediate high-level expression of target genes.\(^\text{27–29}\) LCRs usually regulate multiple genes that are differentially expressed during development or cell-type specification. Transgenic studies have shown that LCRs activate transcription in a position-independent and copy-number dependent manner.\(^\text{27–29}\) The LCRs associated with the human and murine \(\beta\)-globin gene loci have been intensively studied over the last 30 years. The \(\beta\)-globin LCR contains multiple HSs that confer extremely high expression levels to the linked globin genes during development.\(^\text{12,14–16,18}\) Studies using transgenic mice demonstrated that deletion of individual HSs often resulted in a large but variable reduction in globin gene expression, suggesting that the HSs function synergistically.\(^\text{32–34}\) This illustrates that a full complement of HSs is critical for globin activation at ectopic sites, which is significant with respect to using LCRs in gene therapy settings. In contrast, deletions of HS elements from the endogenous mouse globin gene locus revealed that they function additively.\(^\text{35,36}\) The results from the transgenic studies suggested that the individual LCR HSs interact to form a large complex that contacts the globin gene promoters. Recent studies that examined the topology of the murine \(\beta\)-globin gene locus at single allele resolution also revealed interactions between the LCR HSs.\(^\text{17}\) Thus, the \(\beta\)-globin LCR forms a large holocomplex, in which each HS element contributes more or less equally to LCR function in the native chromosomal environment.\(^\text{32,33–37}\)

Transcription originating from \(\beta\)-globin LCR HS2 was reported as early as 1992 by the Tuan laboratory.\(^\text{38}\) Subsequent studies have shown that all \(\beta\)-globin LCR HSs recruit Pol II and exhibit promoter activity.\(^\text{39–41}\) We previously proposed that the \(\beta\)-globin LCR serves as the primary site of transcription complex recruitment, and that transcription at the LCR HSs helps to maintain an accessible chromatin configuration during early stages of erythroid differentiation.\(^\text{42,43}\) We further proposed that at later differentiation stages, transcription complexes are transferred from the LCR to the globin genes.\(^\text{42}\)

SEs are extended chromosomal regions, much longer than traditional enhancers, that are characterized by high accessibility (ATAC-seq or DNase I HSs) as well as by association with histone marks typical for enhancers, including H3K4me1 and H3K27ac.\(^\text{44–46}\) It was shown that some SEs are organized like LCRs in that they contain several HSs that function together to mediate high level target gene activation.\(^\text{47,48}\) It is not clear at this point whether there is a functional distinction between LCRs and SEs. As mentioned before, LCRs have been shown to confer position-independent expression\(^\text{29}\) an activity that, as far as we
1.3. Mediator and the Pol II C-Terminal Domain (CTD) Form Phase Separated Condensates at SEs and LCRs

1.3.1. Mediator Recruits the Unphosphorylated Form of Pol II to SEs and LCRs

Mediator is a large protein complex that functions as a co-regulator for Pol II. It consists of tail, middle, head, and kinase domains that undergo conformational changes during transcriptional activation. Mediator primarily associates with long-distance regulatory DNA elements, including enhancers and SEs. Studies in yeast also demonstrated that Mediator primarily interacts with upstream activating sequences and was only found at core promoters in cells in which Pol II activity was inhibited.

The function of Mediator is not completely understood, but it is involved in the recruitment and assembly of elongation competent Pol II transcription complexes. Thus, while TFIID and other basal transcription factors recruit Pol II to promoters, which are characterized by the presence of consensus basal promoter elements (e.g., TATA, initiator, INR, and downstream promoter element, DPE), the Mediator may be the primary protein complex that governs recruitment and transcription initiation at enhancers. Mediator functionally links transcription factors with components of the basal Pol II transcription machinery. Mediator interacts with the Pol II CTD as well as with basal transcription factors (e.g., TFIID, TFIIH, and TFIID) and elongation factors (e.g., TFIIIS) and regulates enhancer/promoter contacts. Mediator regulated interactions between enhancers and promoters as well as the association of Mediator with components of the preinitiation complex (PIC) are transient. Furthermore, for at least a subset of gene loci Mediator recruits a poised Pol II transcription complex that lacks Kin28, a subunit of TFIH responsible for the phosphorylation of serine-5 of the CTD.

1.3.2. The Pol II CTD Is Phosphorylated During Transcription and Dephosphorylated After Release From SEs and LCRs

The CTD is a long, relatively unstructured scaffold of the largest Pol II subunit. It consists of the heptad Tyr, Ser, Pro, Thr, Ser, Pro, Ser, a motif repeated more than 50 times in humans. All the residues of the heptad except for the prolines are subject to phosphorylation. Ser2 and Ser5 phosphorylation has been extensively studied and shown to play important roles in the transcription cycle. The unphosphorylated form of Pol II is recruited to promoters and interacts with Mediator and TFIID. Transcription initiation is accompanied by Ser5 phosphorylation, which is carried out by the Kin28/CDK7 subunit of basal transcription factor TFIH. Ser5 phosphorylation contributes to the release of Pol II from the transcription start site (TSS) by disrupting interactions with the Mediator and provides a recognition motif for the recruitment of the negative transcription elongation factors DSIF (DRB sensitivity inhibitory factor) and NELF (negative elongation factor). These two proteins temporarily halt transcription to allow capping of the 5’end of the mRNA. Shortly thereafter, pTEFB (transcription elongation factor B) phosphorylates DSIF and Ser2 leading to the dissociation of NELF and the conversion of DSIF into a positive transcription elongation factor. During transcription elongation there is increased phosphorylation of Ser2, which attracts mRNA processing activities, and decreased phosphorylation of Ser5.

There are several phosphatases capable of removing phosphates from the CTD. RNA Polymerase Associated Protein 2 (RPAP2), the human homologue of yeast Rtt1, is a Ser5 phosphatase that appears to play a role during the transition from transcription initiation to elongation. There is an interesting connection between RPAP2 and SEs. A recent study found that depletion of the RNA Polymerase Associated Protein 1 (RPAP1) caused a reduction of total Pol II and Ser5 phosphorylation mainly at SE regulated genes. RPAP1 is required for the interaction between Mediator and Pol II and for the recruitment of RPAP2. Because Mediator is found primarily at enhancers and prominently at SEs it is reasonable to speculate that RPAP1 and RPAP2 regulate the phosphorylation status of Pol II during early transcription initiation and perhaps after transcription abortion and termination at SEs and LCRs.

1.3.3. Phase Separation at LCRs and SEs Facilitates Assembly of Elongation Competent Transcription Complexes

Recent studies demonstrate that high concentrations of Pol II CTD and Mediator, through multivalent interactions, form condensates leading to the formation of liquid droplets, a process referred to as phase separation. Formation of liquid droplets is mediated by intrinsically disordered low complexity sequence domains (LCDs) present in the Pol II CTD and in the Mediator associated cofactor Brd4. Phase separation has been observed at SEs, a feature that is likely due to high levels of Pol II and Mediator recruited to these complex DNA-regulatory elements. Cho et al. demonstrated that SE target genes associate with the condensates during activation of transcription. Formation of Mediator and Pol II aggregates (condensates) is inhibited in cells exposed to 1,6-hexanediol or to an inhibitor of the Mediator associated cofactor Brd4. Inhibition of phase separation/condensation reduced the levels of Pol II and Mediator at the SEs and diminished transcription of target genes. Interestingly, Boechnig et al. showed that phase separation occurred only with the
unphosphorylated form of Pol II and that phosphorylation of the CTD releases Pol II and allows transcription elongation at target genes. This is consistent with previous studies demonstrating that unphosphorylated Pol II interacts with LCDs and that this interaction is disrupted by CTD phosphorylation. These data support a previously published model suggesting that phase separation is an important part of SE mediated gene regulation.

2. Hypothesis

2.1. Elongation Competent Pol II Transcription Complexes Are Assembled at LCRs and SEs and Transferred to High Affinity Target Promoters During Transient Looping Interactions

We propose that the multiple HSs associated with LCRs and SEs efficiently recruit Pol II and other components of the transcription complex, which are then transferred to high affinity target promoters during transient looping interactions. Efficient recruitment of Pol II and assembly of transcription complexes is the result of two factors: high fractional accessibility at the HSs; and a large number of transcription factors and Mediator that bind to these sites and contact components of the Pol II transcription machinery. The high local concentration of transcription factors and co-regulators at LCRs and SEs provides an optimal environment for the assembly of elongation competent Pol II. Transcription initiation within LCR or SE associated HSs occurs bidirectionally and due to instability leads to frequent abortion and release of elongation competent Pol II transcription complexes. The initial transcribing complexes may not be phosphorylated at Ser5 because Mediator may recruit a Kin28 deficient TFIIH subcomplex. However, Ser5 that is phosphorylated during initiation and early elongation steps would be dephosphorylated by RPAP2, which is recruited to SEs by Mediator and RPAP1. We propose that dephosphorylated but otherwise complete elongation competent transcription complexes are transferred to target promoters during transient loop interactions. Assembly and transfer of transcription complexes is facilitated by phase separation, which contributes to concentrating all activities at SEs and LCRs and may also facilitate the recruitment of target genes to the phase separated domains. Because of the multiple HSs associated with SEs and LCRs, the transient interactions allow a large number of elongation competent transcription complexes to be transferred to the strong basal promoter elements (TATA, INR, and DPE) at the target genes, which is consistent with the bursting phenomenon. It is notable that target genes of Mediator, and consequently of SEs, contain consensus TATA motifs. Accordingly, the human β-globin LCR preferentially activates transcription of the major adult β-globin promoter, which contains a TATA consensus sequence, compared with the minor adult δ-globin gene, which contains a point mutation in the TATA box. It is interesting to note that the presence of strong basal promoter elements, especially the TATA box, increases the transcription burst size, while Mediator levels determine bursting frequencies.

An additional source of elongation competent Pol II may be provided by termination of eRNA transcription mediated by Integrator and other protein complexes. The Integrator plays a role in 3' end cleavage of eRNA transcripts thereby leading to termination of transcription and release of mature eRNA, Pol II, and Integrator complex. The transcription complexes released by Integrator are characterized by the presence of phosphorylated Ser2 and Ser5, which have to be dephosphorylated before transfer to the genes. It is possible that transcription termination of eRNAs by Integrator may prevent formation of long potentially harmful non-coding transcripts. Alternatively, the eRNAs that are released during transcription termination may play a role in the activation of the target genes. For example, the eRNAs could participate in phase separation proposed to be important for enhancer mediated transcription activation.

The prevailing model of LCR and SE function is that the distal regulatory elements recruit Mediator and other co-activators, which either facilitate assembly of Pol II transcription complexes at target promoters and/or promote transcription elongation (“bridging or kissing” models). The recent data on phase separation appear to be consistent with this model. For example, Sabari et al. found that inhibition of phase separation by 1,6-hexanediol led to a reduction of Pol II occupancy at the coding region of an SE target gene but not at the promoter. This is consistent with previous studies on the murine β-globin LCR showing that deletion of the LCR results in defects in transcription elongation. However, we would argue that the data on Mediator and Pol II condensation are also consistent with a Pol II transfer model. In the absence of SE associated phase separation or after deletion of an LCR, the formation of elongation competent transcription complexes at the target promoters is likely very inefficient due to low concentration of the activities required for assembly. Slow assembly of elongation competent transcription complexes accumulates Pol II at the promoters. Elongation competent transcription complexes are efficiently assembled at LCRs and at SEs due to the high concentration of co-activators and Pol II. In contrast to the “bridging/kissing” model, the Pol II transfer model takes into account the fact that SEs and LCRs recruit high levels of Pol II. Especially the data from Boehning et al. suggest that SEs recruit and assemble unphosphorylated Pol II. Transcription associated phosphorylation dissociates Pol II from the phase separated domain at SEs and allow recruitment to target gene promoters during transient loop interactions. This scenario is consistent with observations showing that Mediator recruits RNAP9/CDK7 deficient TFIIH and unphosphorylated Pol II. The published data suggest that there are three different forms of Pol II at SEs that are available for transfer to target gene promoters: unphosphorylated Pol II recruited by Mediator, Ser5 phosphorylated Pol II released during early abortion of transcription and Ser2 phosphorylated Pol II released by Integrator. As mentioned before, the phosphorylated forms will likely have to be dephosphorylated before they are recruited to target gene promoters.

2.1.1. The Human β-Globin LCR Serves as a Center for Transcription Complex Assembly

The Pol II transfer model is summarized in Figure 2 using the human β-globin gene locus as an example. This complex gene
locus consists of five genes that are expressed in a developmental stage specific manner. The adult β-globin gene is expressed at extremely high levels in erythroid cells differentiating from stem cells in the bone marrow. The five genes are regulated by an LCR that harbors five HSs Pol II is recruited to all of the LCR HSs and initiates transcription of eRNA. We propose that transcription complexes are efficiently recruited and assembled at the LCR HSs in the context of phase separated domains. Most of the recruited and assembled Pol II may be unphosphorylated and phosphorylation takes place after transfer to the globin gene promoters. Phosphorylated Pol II, especially Pol II released by integrator, will be de-phosphorylated before transfer to the globin gene promoter.

The human β-globin LCR has been extensively studied and the proposed model of LCR/SE function is consistent with many of the data accumulated over the last 30 years since its discovery. For example, studies in the endogenous murine β-globin gene locus demonstrated that the LCR HSs function additively and this is explained by each HS providing an equal number of transcription complexes that are transferred to the genes. Moreover, inhibition of transcription elongation at the adult β-globin promoter led to increased Pol II levels at the LCR. Finally, Pol II transfer to the β-globin promoter has been recapitulated in vitro using immobilized LCR constructs. The Groudine laboratory demonstrated that there is a decrease in the recruitment of Pol II at the adult β-globin gene and an even more dramatic decrease in elongation activity of recruited transcription complexes in the absence of the murine β-globin LCR. This is consistent with our proposed model and suggests that elongation competent Pol II transcription complexes are more efficiently assembled and primed at the LCR HSs, which may represent an erythroid-specific transcription factory. Furthermore, the Grosveld laboratory demonstrated that transcription complexes associate with genes in the context of transcription factories and that transcription elongation occurs away from these factories. This is consistent with transcription complex recruitment at SEs and LCRs, transient contact with the target genes, transfer of Pol II to the promoters, and transcription of the genes proceeding away from the phase separated domains. Moreover, the observation that enhancer mediated target gene transcription occurs in bursts is consistent with transient enhancer/promoter interactions during which elongation competent Pol II is transferred to the genes.

3. Conclusion and Outlook

Accumulating evidence suggests that SEs and LCRs efficiently recruit and assemble elongation competent transcription complexes. We propose that abortion and termination of transcription at SEs/LCRs releases a large pool of transcription complexes that are efficiently captured by strong basal promoter elements at target gene promoters during transient interactions. The Pol II transfer model may also apply to single enhancers that are transcribed and activate transcription by transient looping interactions with target gene promoters. However, as mentioned previously, enhancers may activate transcription by different mechanisms. For example, enhancers located in close proximity to promoters are unlikely to function by the proposed Pol II transfer mechanism. In the future, it will be important to investigate in more detail the role of the Mediator and the Pol II CTD phosphorylation status at SEs and at promoters. Furthermore, it will be important to design additional experimental settings that will support or refute the Pol II transfer model. For example, inhibiting transcription termination at SEs and LCRs should reduce Pol II at target genes and accumulate it at the distal elements. Likewise, introducing mutations into basal promoter elements, particularly the TATA box at target genes should prevent transfer to the genes and should increase the levels of Pol II at the SEs and
LCRs. Another potential strategy to test the Pol II transfer model is to express a fluorescently labeled Pol II in an inducible manner. If combined with an inducible SE driven transcription system in which the SE and target gene are located far away from each other, Pol II could be tracked by combining fluorescent microscopy and super-resolution DNA-FISH during the activation process.[85]

**Abbreviations**

ATAC-seq, assay for transposase-accessible chromatin using sequencing; ChIP, chromatin immunoprecipitation; CTD, C-terminal domain; DSIF, DRB-sensitive inhibitory factor; eRNA, enhancer RNA; HS, hypersensitive site; INR, initiator; LCD, low complexity sequence domain; LCR, locus control region; mRNA, messenger RNA; NELF, negative elongation factor; Pol II, RNA polymerase II; pTEFB, transcription elongation factor B; RPAP, RNA polymerase associated protein; SE, super-enhancer; TSS, transcription start site.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

mediators, phase separation, RNA polymerase II, super-enhancer, transcription regulation

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