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Distal enhancers loop to proximal enhancers, not to promoters

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Although enhancers activate transcription from long distances, they stimulate transcription only through short-range interactions with the RNA polymerase II machinery. I posit that action at a distance is mediated by loops between distal and proximal enhancers that thereby bring proteins associated with distal enhancers near promoters.

Eukaryotic enhancers are typically defined as genetic elements that activate transcription by RNA polymerase II (Pol II) when located at long distances from the promoter. Multiple such distal enhancers — with different regulatory specificities — can activate an individual promoter and, hence, an individual gene. Enhancers contain multiple binding sites for activator proteins, which co-localize to regions of 100–300 base pairs, thereby imposing combinatorial activation of transcription needed for the extraordinary diversity of gene-regulation patterns.

Transcription is directly activated by promoter-proximal enhancers

Proximal enhancers, which also consist of multiple binding sites for activator proteins, are typically located 50–300 bp upstream of promoters. Proximal and distal enhancers are mechanistically similar, but they are distinguished by their location relative to promoters: distal enhancers are >500 bp and often many kilobases upstream or downstream of genes. Genome wide, individual activator proteins bind to both proximal enhancers and distal enhancers.

Proximal enhancers are mechanistically distinct from their adjacent promoters, which are recognized by the Pol II transcription machinery. At both proximal enhancers and distal enhancers, activators (often with recruited co-activators) perform three distinct functions: chromatin modification; interaction with the basic Pol II machinery; and DNA looping between physically separate genomic regions.

Enhancer-bound activator proteins recruit nucleosome-remodelling and histone acetylase complexes, which leads to nucleosome depletion and histone hyperacetylation at the enhancer[1]. In addition, enhancer-bound activator proteins invariably generate enhancer RNAs that initiate bidirectionally, typically in a non-sequence-specific manner, from the edges of the nucleosome-depleted region that they created[2].

Enhancer-bound activator proteins can also interact directly with components of the Pol II transcription machinery through their activation domains. Mediator, a multiprotein complex that directly interacts with Pol II[3], is the main target of activator proteins, and it acts as a dynamic bridge between enhancers and promoters (Supplementary Fig. 1). Some activators interact directly with TAF subunits of the TFIID complex that directly binds promoter sequences[4].

The communication between enhancer-bound activators and Mediator (or TFIID) bound to promoters occurs only over short distances. In transient transfection experiments, the enhancer of the gene encoding β-interferon (*IFNB1*) strongly activates transcription from the *IFNB1* promoter when the two elements are 75 bp apart, but activation is diminished at a distance of 220 bp and is essentially eliminated at >500 bp (ref. 5). At the endogenous locus encoding β-globin (*HBB*) in GATA1-deficient cells, the enhancer stimulates transcription when located close to the promoter but not when located far from it[6]. Similar results are observed in systematic distance experiments between the yeast *GAL* enhancer and *his3* promoter, and activation does not occur when the enhancer is downstream of the promoter[7]. In all these cases, promoters are stimulated directly by proximal enhancers, but not by distal enhancers.

Enhancer–enhancer interactions mediate activation at long distances

The short-range nature of direct enhancer–promoter communication appears to conflict with the common view that enhancers act on promoters at long distances and with the numerous examples of long-distance looping between enhancers and promoters. The common misconception that enhancer–promoter loops mediate long-distance activation reflects semantic confusion between enhancers, which are activator-binding sites, and promoters, which are bound by the basic Pol II machinery. I argue that instead, long-distance loops arise from interactions between proteins associated with distal and proximal enhancers.

In transient transfection experiments, the *IFNB1* enhancer stimulates transcription when located 2.3 kb upstream from the promoter of the gene encoding thymidine kinase, but only if the promoter-adjacent region containing sites for the transcriptional activator SP1 is present[5]. Thus, long-range activation requires a functional connection between proteins bound at the distal enhancer and at the proximal enhancer that contains SP1 sites. Strikingly, the requirement for SP1 sites can be bypassed by artificially connecting the distal and proximal regions through interaction of λ repressor dimers at target sites mediated by their tetramerization looping domain[5].

At the endogenous *HBB* gene, the locus-control region (more recently termed a super-enhancer) does not stimulate transcription when the proximal enhancer is genetically inactivated through knockout of GATA-1 (ref. 6). However, an artificial zinc-finger protein that binds sequences near the inactivated proximal enhancer can activate *HBB* transcription, but only if it contains the SA multimerization domain of the transcription cofactor LDB1 (ref. 6). At the endogenous locus, LDB1 interacts with LMO1, a protein associated with, but not directly bound to, both the proximal enhancer and the distal super-enhancer in a manner that depends on binding of GATA-1 to the

enhancers. Thus, action at a distance at the *HBB* locus involves looping between proximal and distal enhancers, which is crucially mediated by the LDB1 SA domain[6]. In other experiments, long-range activation scales with genomic distance[8], and enhancers can cooperate over large distances, possibly reflecting loops between distal enhancers[9].

In *Drosophila melanogaster*, 'tethering elements' have an analogous role in looping between proximal and distal enhancers, except that they do not activate transcription on their own[10]. Tethering elements are located near distal enhancers and promoters, and they are bound to GAGA sequences by GAF, a protein that recruits nucleosome-remodelling complexes but seems to lack an activation domain. By analogy with LDB1, GAF contains a BTB/POZ oligomerization domain that mediates looping between tethering elements. Mutations of the tethering elements or the BTB/POZ domain block looping and transcription activation, again indicating that distal enhancers cannot mediate long-range activation on their own[10]. In yeast, artificial introduction of GAF-mediated loops far from the promoter permits long-range activation by a distal enhancer[11].

In accord with the conclusion that activators bound to distal enhancers do not form transcription-competent loops by interacting with Mediator (or TFIID) at the promoter, looping on a genomic scale is only subtly diminished when Pol II transcription is eliminated through inactivation of Mediator or TFIID[12,13]. By contrast, loss of LDB1 (ref. 14) or GAF[10] severely reduces the occurrence of a subset of loops in a manner that does not involve the insulator protein CTCF, cohesin, or loop extrusion; hence, the LDB1-dependent and GAF-dependent loops are distinct from loops involved in topologically associating domains[14].

Thus, protein–protein interactions that mediate looping are distinct from those that mediate transcription activation, although both types of interactions are crucial for enhancer action at a distance (Supplementary Fig. 1). The requirement that enhancer–enhancer loops mediate activation at a distance imposes regulatory specificity; otherwise, distal enhancers would potentially loop to all promoters that are bound by Mediator and TFIID. Although it has not yet been observed, it remains possible that some super-active enhancers or super-enhancers stimulate transcription solely by interacting with Mediator over longer distances.

Transcription-activation mechanisms

Upon looping to proximal enhancers, activator proteins bound at distal enhancers are brought close to the promoter, thereby allowing their activation domains to mediate short-range interactions with Mediator (or TFIID) (Supplementary Fig. 1). However, activators bound at distal enhancers must stimulate transcription from the promoter, beyond the level achieved by activators bound at proximal enhancers alone. Proteins associated with distal and proximal enhancers might synergistically activate transcription through multiple activation domains near the promoter. Alternatively, as exemplified by GAF, proteins bound near proximal promoters could recruit chromatin-modifying enzymes but not Mediator, in which case activation would require other looped proteins bound to or near the distal enhancer. Looping interactions could also permit cooperative binding of activators to separated target sites, leading to increased transcription.

The physical nature of long-range looping interactions

Looping in *Escherichia coli* involves bi-molecular interactions between transcription-regulatory proteins bound to separated target sites. By contrast, looping by LDB1 (ref. 6) and GAF[10] involves multimerization domains that generate higher-order complexes and multi-locus networks. A multimerization domain of the DNA-binding transcription factor FOXP3 also mediates looping, although its biological role has yet to be determined[15]. Multi-molecular interactions are probably stronger than bimolecular interactions, which might explain why loops in eukaryotes occur at much larger distances than those in prokaryotes. Activator–Mediator interactions are also bimolecular and rather weak, which might explain their short-range effects. Finally, crucial interactions may not involve simple adhesive surfaces but may instead involve condensate-forming domains. Condensates could link many genomic regions into a hub that contains high concentrations of key transcription-regulating proteins, thereby increasing transcription activation.

Conclusion and future perspective

Although looping between proteins associated with enhancers explains the mechanism and specificity of regulated gene activation at a distance, the dynamics of loop formation and the relationship to transcription activation are unclear. Loops are typically portrayed as stable entities, but there is accumulating evidence that they are dynamic and relatively infrequent. In addition, the temporal relationship between loop formation and transcription activation is controversial, and it may differ among the enhancers and promoters involved. Mechanistic and structural studies are needed to identify proteins that mediate loops between proximal and distal enhancers and to address how such loops mediate transcription activation.

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Additional information

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