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Review Article

Functional genomic assays to annotate enhancer–promoter interactions genome wide

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Abstract

Enhancers are pivotal for regulating gene transcription that occurs at promoters. Identification of the interacting enhancer–promoter pairs and understanding the mechanisms behind how they interact and how enhancers modulate transcription can provide fundamental insight into gene regulatory networks. Recently, advances in high-throughput methods in three major areas—chromosome conformation capture assay, such as Hi-C to study basic chromatin architecture, ectopic reporter experiments such as self-transcribing active regulatory region sequencing (STARR-seq) to quantify promoter and enhancer activity, and endogenous perturbations such as clustered regularly interspaced short palindromic repeat interference (CRISPRi) to identify enhancer–promoter compatibility—have further our knowledge about transcription. In this review, we will discuss the major method developments and key findings from these assays.

Introduction

A promoter is a DNA segment where stable transcription usually initiates. This process is highly regulated by *cis*-regulatory elements, including enhancers that activate transcription. Multiple theories about how promoters and enhancers interact were introduced (1–4). These theories are based on a common assumption that when enhancers are present in the close vicinity of different promoters, they specifically interact and elevate the expression of their target promoters. This process is also called enhancer–promoter interaction (EPI). It is of overriding importance to experimentally pinpoint which pairs of promoters and enhancers interact to increase our understanding of the rules governing this compatibility.

Discoveries from multiple perspectives are contributing to a thorough dissection of the compatibility rules. One of these major contributors is the finding of specific compositions of histone marks that are associated with enhancers (5), as they provide clues for genome-wide identification of enhancers (6,7) and thus enable at-scale experimental testing of EPI. Recent studies have also shown that calling distal divergent transcription signals (8,9) captured by assays like Global Run-On (GRO)/Precision nuclear Run-On (PRO)-cap help improve the specificity of enhancer identification (10,11).

The availability of comprehensive enhancer and promoter maps motivates the development of assays that are capable of high-throughput capture of EPIs. In this article, we review the utilization of chromatin capture assays, ectopic reporter assays and endogenous perturbation assays in identifying EPIs. We also summarize the cutting-edge discoveries led by these assays.

Nascent RNA transcriptome identifies enhancer accurately

To study the interactions between promoters and enhancers, it is pivotal to first identify the active promoters and enhancers in the genome. While promoters are easier to locate, identifying enhancers is a more challenging job since the transcribed RNAs at enhancers are usually unstable, and these RNAs do not encode protein. Previously, epigenomic signatures have been employed to locate potential enhancer candidates, with histone marks H3K27ac7 and H3K4me1 as two key factors (12). To achieve higher accuracy, multiple histone marks have been combined in ChromHMM (13) for a more systematic deduction of the enhancers. Other factors such as regions of open chromatin and CCCTC-binding factor (CTCF) binding were also incorporated to better define enhancers (14). The major drawbacks of these histone-based assays include the requirement of multiple experiments to detect different histone marks accurately and the relatively low specificity of identifying a functional enhancer (8,10). Recently, the divergent transcription of enhancer RNAs has been found to be one of the best single marks to identify enhancers (8,10), even without relying on other chromatin marks. The emerging technology to detect nascent RNA transcripts and their respective transcription start sites in a genome-wide manner enables the efficient identification of most active enhancers (15–18). Among these technologies, with sufficient high-quality materials, GRO/PRO-cap was found to be the best to detect enhancers accurately (11). In fact, the nascent RNA transcription could also be used to define the boundary of a functional enhancer unit (Fig. 1) (10).

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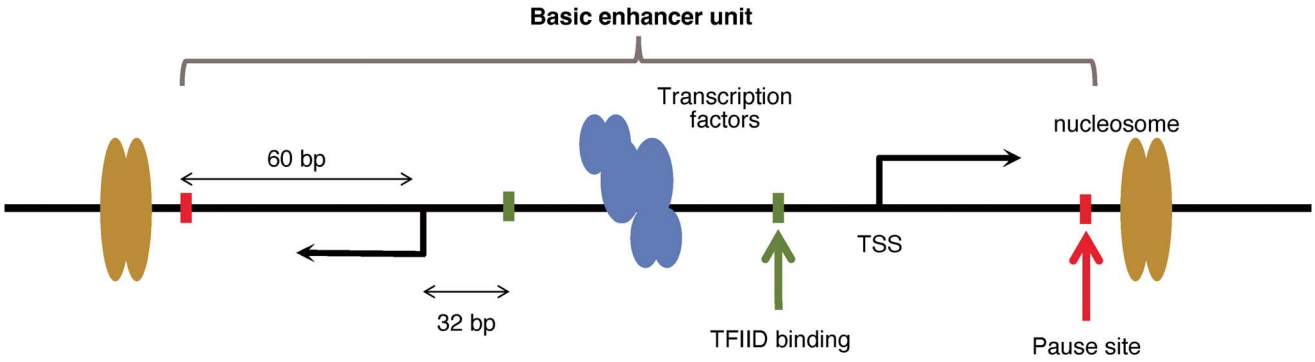

Figure 1. The architecture of an enhancer unit. The divergent transcription marks the active enhancer and its core components and boundary. Core promoter motifs are located between ~32 bp upstream of the TSS (TFIID binding) and ~60 bp downstream of TSS (pause sites for RNA polymerase).

Chromatin conformation capture assay reveals dynamics of promoter–enhancer interactions within topological associated domains

Chromatin folding allows *cis*-regulatory elements that could be up to million base pairs away from their target promoters to be brought in contact with them. Chromosome conformation capture (3C) (19) has been employed to study the dynamics of chromosome architecture in yeast cells during meiosis. The strategy relies on the ligation of proximal DNAs. The ligation frequency of these DNA segments reflects their interaction probability. Later, with the use of high-throughput sequencing and various DNA amplification techniques, the field has moved from targeting a single region in 3C to a many-to-one region in circular chromosome conformation capture (4C) (20) and many-to-many regions in chromosome conformation capture carbon copy (5C) (21). Hi-C represents one of the milestones for studying chromosomal architecture in a genome-wide manner (22), enabling a more comprehensive picture across interactions in the whole genome and furthering our understanding of the topologically associating domains (TADs). CTCF and cohesin are critical players in defining this fundamental chromatin architecture component through loop extrusion (3). On the one hand, such boundaries facilitate intradomain interactions. On the other hand, the boundary also serves as an insulator that prevents regulatory elements from outside from interacting with the promoters within the TADs (Fig. 2). Disrupting them causes 'unlooping' across the chromatin and leads to genome-wide disruption of enhancer and promoter pairs (23). CTCF-binding sites (CBS) that define the boundaries of TADs, when in clusters, could also cooperate redundantly to increase robustness such that the interactions within the TAD remain unaffected even if individual CBS is disrupted (24). Disruption of a single TAD could also lead to aberrant gene expression during development and eventually exceptional phenotypes.

The interaction between promoters and enhancers is a very dynamic process. The interaction of enhancer and promoter is not always a one-on-one situation. Within a TAD, enhancers and promoters could freely interact with enhancer–promoter, promoter–promoter and even enhancer–enhancer pairs (25). In fact, many interacting promoters in the genome are inactive, and some promoters could act as silencers to lower the transcription level of their interacting partner (26). Several optimizations have been made for higher resolution, including Micro-C (27) and in situ Hi-C (28). In addition, to focus on chromatin interactions around proteins of interest, the chromatin interaction assay is integrated with chromatin immunoprecipitation (ChIP). These methods include ChIA-PET (29), HiChIP (30) and PLAC-seq (31). For example, a HiChIP assay targeting H3K27ac, a histone mark for active enhancers, has revealed cell-type specific EPIs at 1-kbp resolution (32). Rather than relying on protein binding, regions of interest could also be specifically targeted by probing using a pool of oligonucleotides, as used in the Capture-C (33). Efforts have also been made to study promoter-centered interactions (34) and enhancer-centered interactions (35). The oligonucleotides pool must be designed based on existing knowledge of target promoter or enhancer regions. Rather than selecting certain proteins of interest or regions of interest, chromatin interactions among open chromatin regions are of great interest since it is where interacting enhancers and promoters occur. To focus more on interactions in open chromatin, various methods have been developed to integrate the chromatin capture assay with existing sequencing methods that assess chromatin accessibility, such as Trac-looping (36) and HiCAR (26), Ocean-C (37), HiCoP (38) and NicE-C (39).

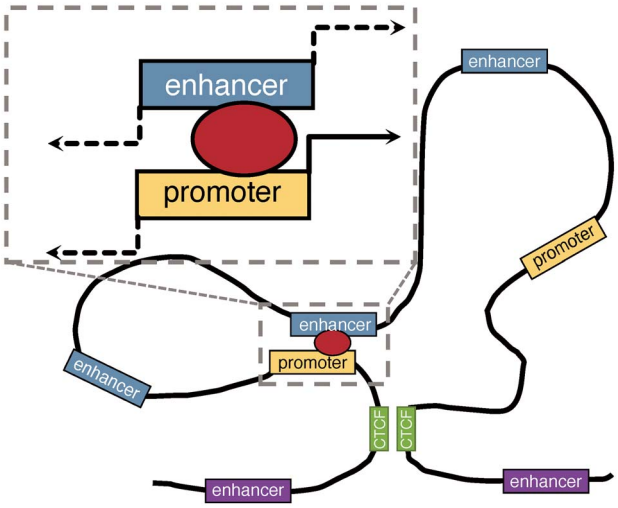

Figure 2. Illustration of promoter–enhancer interactions within TAD. Across the genome, architectural proteins such as CTCF define TADs to facilitate interaction between elements inside them. CTCF interaction is usually more stable, while interaction between enhancers and promoters is more dynamic. The TAD also serves as an insulator such that enhancers and promoters outside the TAD will have minimal chance to interact with those within the TAD. When gene transcription occurs, unstable and low-abundance transcription also occurs at the other side of the promoter, and in both directions of the enhancer. These divergent transcripts mark the active enhancers in the genome as shown in Figure 1.

In certain circumstances, promoters can interact with multiple enhancers at the same time (40). Modified from the previous ligation-based approach, several methods have been developed to obtain multi-way chromatin contacts, including C-walks (41), Tri-C (40) and Multi-contact 4C (42). Some innovative non-ligation-based methods are also introduced to resolve the multi-way chromatin contacts. In genome architecture mapping (GAM) (43), the random orientation of slices on nuclei is extracted such that proximal DNA segments co-occur more often in different slices. In the split–pool recognition of interactions by tag extension (SPRITE) (44) and ChIA-drop (45), chromatin is crosslinked, followed by fragmentation. In SPRITE, a repeated split-and-pool strategy with the sequential addition of tags is employed such that proximal DNA segments will be attached with a unique series of ligated tags. In ChIA-drop, DNA segments are partitioned using a microfluidic device, achieving the labeling of proximal DNA segments with the same unique tags. To complement the limitation of sequencing-based methods, a multi-color live-cell imaging technique was used to study in vivo promoter-enhancer interactions. This uncovered that gene transcription activation at promoters requires sustained proximity between the promoter and enhancers (46), and the level of transcription is directly related to the contact frequency between enhancer and promoter (47).

Ectopic reporter experiments reveal that core promoter composition and intrinsic activity of promoters affect EPI

A new era started when Banerji *et al.* integrated the SV40 DNA sequence and β-globin gene into a plasmid and observed hundred times boost in β-globin production driven by the SV40 DNA sequence (48). DNA sequences that enhance transcription were later termed enhancers. Testing the function of DNA sequences outside their endogenous genomic contexts by reading the expression changes on a reporter is called ectopic reporter assay. Starting from the 1990s, ectopic assays were used to identify DNA sequences that determine the compatibility between enhancers and promoters. In these experiments, researchers first noticed that enhancers might only work with certain types of promoters (49–51). This selectivity is partially explained by the preference of enhancers toward promoters with different core promoter elements. For example, an enhancer located upstream of the human myoglobin gene interacts with the myoglobin promoter but not the SV40 promoter because of the differences in their TATA-boxes (52). Similarly, the preferences of enhancers toward promoters with different core promoter elements (TATA-box and downstream promoter element, DPE) were observed in *Drosophila* (53,54). However, to what extent enhancers' preferences over different core promoter elements affect EPI, especially in humans, is still largely unexplored.

The maturity of high-throughput sequencing techniques allows for the development of parallel testing of multiple enhancers and their impact on transcription, like massively parallel reporter assay (55–58) and self-transcribing active regulatory region sequencing (STARR-seq) (59). In these assays, different enhancers interact with a fixed minimal promoter, and the changes in reporters' expression levels are considered enhancers' activities. To test the intrinsic compatibility between promoters and enhancers, instead of only swapping the enhancer candidates, the minimal promoter also needs to be replaced by other promoters of interest (Fig. 3A). The first attempt happened in *Drosophila melanogaster* S2 cells and ovarian somatic cells (60), where two parallel STARR-seq experiments were performed with different promoters: one used the core promoter from a housekeeping gene *RpS12* (has TCT motif) and the other used a modified developmental core promoter (has TATA-box, initiator, motif 10 element and DPE). This study identified distinct populations of enhancers that work with either the housekeeping or the developmental promoter. It also showed that the binding of transcription factors Dref (for the housekeeping promoter) and Trl (for the developmental promoter) contributes to the selectivity between enhancers and promoters.

More recent studies performed in mouse embryonic stem cells (61) and human K562 cells (62) pushed the throughput to a higher limit (up to 604 268 enhancer–promoter pairs), thus enabling a more comprehensive view of the underlying determinants of EPI. Based on their collected data, both studies suggested that promoters' intrinsic (or basal level) activity plays an important role in determining enhancer–promoter compatibility; promoters with high basal activity were less responsive to enhancers. However, there are some contradictions in these two studies. For instance, the K562-based study (62) showed that enhancers with high intrinsic activity could boost the expression with a broader spectrum of promoters. Similar to the *Drosophila*-based work (60), this study noticed a dichotomy of compatibility between housekeeping and non-housekeeping promoters and further pointed out that promoters with strong intrinsic activity (less responsive to enhancers) are usually from housekeeping genes, with good binding signals from transcription factors of the ETS family, YY1, among other transcription factors (TFs). In contrast, the mouse Embryonic Stem Cell (mESC)-based study (61) suggests the independence between a promoter's class (housekeeping or not) and EPI.

Several factors affect the credibility of ectopic results (see also Cooper *et al.* this volume). First, comparative studies about in-genome and episomal ectopic assays with fixed minimal promoters have shown that genomic context can affect the absolute measurements of enhancer activity (63–65). In the future, a revisit of current conclusions will be of great importance with improved versions of in-genome ectopic reporter assays (63,64) that are capable of swapping both enhancers and promoters (Fig. 3A). Second, the architecture of enhancers is still not completely understood (10), and the incomplete cloning or synthesis of candidate enhancers may confound the interpretation of the compatibility rule. Last, the distance between enhancers and promoters on the ectopic cassette is usually much closer than the actual linear distances in their original context; thus, regulation through conformation constraint may be skipped in these settings. One way to address this is to combine ectopic reporter assays with downstream high throughput clustered regularly interspaced short palindromic repeat (CRISPR) validation, such as recently done (66).

Genome-wide endogenous perturbation studies indicate the general rule of EPI

Endogenous perturbations of candidate enhancer regions followed by detecting the changes in gene expression is an essential strategy for identifying enhancers and their target genes. These perturbations can be introduced by knocking out candidate enhancers. For example, using transcription activator-like effector nuclease (TALEN), researchers identified an enhancer that interacts with *BCL11A* in human erythrocytes (67). CRISPR/Cas9, because of its flexibility, is becoming a more popular

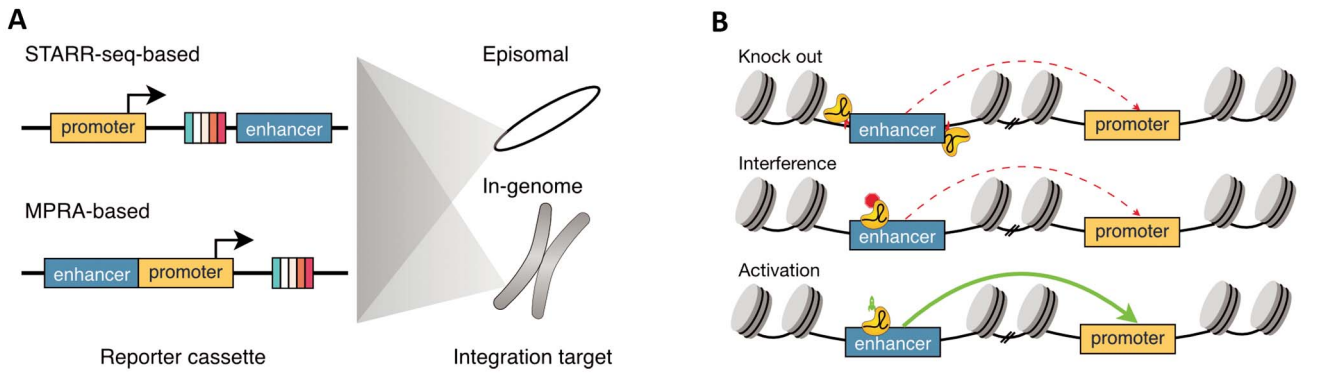

Figure 3. Illustration of ectopic reporter assays and endogenous perturbation methods for identifying EPIs. (**A**) Ectopic assays rely on constructing reporter cassettes with promoters and candidate enhancers of interest. Barcodes (BCs) are usually added to the cassettes to make contributions from different elements distinguishable. When integrating the reporter cassettes into plasmids, the following testing is considered episomal, while if the cassettes are integrated into chromosomes, the testing is considered in-genome. (**B**) The overview of three types of endogenous perturbation methods (CRISPR-based). For interference-based methods, the red octagon represents the fused repressor. For activation-based methods, the green rocket represents the fused activator.

choice for endogenous perturbations (Fig. 3B). Multiple enhancer targets have been successfully identified with this method (68–75), including enhancers of globin genes (69,75), *CDKN1A* (70) and *Myc* (76).

The methods mentioned above have bottlenecks in testing scalability from two aspects: first, they are usually coupled with RT-qPCR (68,77), Fluorescence-Activated Cell Sorting (FACS) (71,75) or phenotypic selection (70,72,74,78), which limits the number of target genes that can be queried. Second, the precise knockout of enhancer regions with CRISPR/Cas9 usually requires the concurrent introduction of two guide RNAs (gRNAs), making it relatively inconvenient when perturbing many enhancers (78,79). One way to improve the perturbation throughput is to use catalytically inactive Cas9 protein (dCas9). When fused to repressive chromatin modifiers, like KRAB, it induces heterochromatin around the target sites (80,81), and this is termed CRISPR interference (CRISPRi); dCas9 can also be fused to transcription activators, such as a hybrid VP64-p65-Rta tripartite activator (82), and the system is called CRISPR activation (CRISPRa). CRISPRi (77,78,83) and CRISPRa (84) work with a single gRNA, which makes the large-scale perturbation of enhancer candidates an easier task (Fig. 3B). The maturation of single-cell CRISPR screening (85–88) further enabled the simultaneous identification of enhancers and their targets at a larger scale (89–92). Besides, many efforts have been made to increase the statistical power for detecting gene expression changes. For instance, leveraging the synergy between multiplex CRISPRi and single-cell sequencing, Gasperini *et al.* identified 664 *cis* enhancer–gene pairs in human K562 cells (90); Schraivogel *et al.* used semi-nested target amplification and successfully tested the regulatory relationships between 1778 enhancers and 149 genes (92).

Based on the identified EPIs from large-scale endogenous perturbations, we can draw three preliminary conclusions: first, about 73–95% of surveyed enhancers only regulate one gene (83,90–92). Second, although the linear distance was widely used in assigning enhancers to their target genes, 16–48% of the enhancers do not regulate their closest expressed genes (90–92). Third, substantially elevated contact frequency was observed when overlaying validated EPIs with conformation capture experiments (90,92).

EPIs identified by endogenous perturbations are considered highly credible and have been widely used as the ground truth in building in silico tools for predicting EPI (83,93,94). However, complete avoidance of false positive and negative EPIs is still challenging. For example, shadow enhancers (95,96), which are redundant enhancers that regulate the same gene, play an important role in the course of development. When perturbing one of the shadow enhancers, the disruption may be compensated by other enhancers and thus lead to false negatives. Technical issues, such as targeting efficiency (97) and specificity (98–100), can also affect the reliability of identified EPIs. For interference- and activation-based methods, the perturbation affects relatively broader regions (usually within 200–900 bp), and ambiguity can come from other enhancer candidates in the vicinity of the perturbed target (77,83,101). It may be helpful to reduce false positives by overlaying CRISPRi- and CRISPRa-identified enhancers with epigenomic evidence such as histone marks (14) and divergent transcription (11).

Future perspectives

Chromatin conformation capture techniques blossomed our general understanding of how enhancers and promoters interact and enabled the invention of numerous *in silico* EPI prediction tools (102). The recent development of ectopic reporter assays and endogenous perturbation assays further makes large-scale identification of enhancer–promoter pairs with high accuracy possible. Preliminary conclusions from these studies suggest that important roles the intrinsic activity of promoters and enhancers may play in the determination of EPIs; however, the bridge between the sequence composition of enhancers and promoters and their activity is still missing. With the continuous integration between biological science and data science, the rapid accumulation of these functional characterization data promises a more in-depth understanding of the sequence determinants of EPIs in the near future.

Variants residing in enhancers can affect the functionality of enhancers and thus influence the expression of target genes. Quantitative trait loci (QTL) analysis identifies variants that are associated with gene expression changes, and when overlaying these variants with enhancer candidates, it serves as a high-throughput approach to identify EPIs. Considering the increasing availability of high-quality enhancer maps, QTL-based approaches will certainly deepen our understanding about EPIs. A comprehensive review on quantitative trait locus approaches

for identifying non-coding variants can be found in Bykova *et al.* this volume.

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