Shaping the nebulous enhancer in the era of high-throughput assays and genome editing

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Abstract

Since the first discovery of transcriptional enhancers in 1981, their textbook definition has remained largely unchanged in the past 37 years. With the emergence of high-throughput assays and genome editing, which are switching the paradigm from bottom-up discovery and testing of individual enhancers to top-down profiling of enhancer activities genome-wide, it has become increasingly evidenced that this classical definition has left substantial gray areas in different aspects. Here we survey a representative set of recent research articles and report the definitions of enhancers they have adopted. The results reveal that a wide spectrum of definitions is used usually without the definition stated explicitly, which could lead to difficulties in data interpretation and downstream analyses. Based on these findings, we discuss the practical implications and suggestions for future studies.

Keywords

Enhancers, cis-regulatory elements, transcriptional regulation,

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[*] Equal contributions

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Introduction

The concept of transcriptional enhancers originated from three papers published in 1981 that studied gene expression in Simian Virus 40 (SV40) [1–3]. These studies identified a 72bp repeat sequence around 150-200bp upstream of the early genes of SV40 that was necessary for their transcription. In one of the studies, it was further discovered that this "enhancer" element (term coined in Banerji et al. (1981)) could greatly enhance reporter gene expression at various distances from the gene no matter it was placed upstream or downstream of it [1]. The enhancer was also found to be *cis*-acting, in that covalent linkage to the recombinant was required for the stimulation of expression. Based on these initial findings, enhancers have since been commonly defined as *cis*-regulatory elements that can enhance the expression of target genes in a distance- and orientation-independent manner [4–7].

Research in enhancers has been accelerated in recent years by the emergence of new technologies for identifying and characterizing enhancers [8–14], quantifying enhancer activities in particular cell and tissue types [15–18], determining enhancer targets [19,20,29,21–28], and evaluating the functional consequence of genomic [30–37] or epigenomic [31,38–44] perturbation of enhancers in normal conditions and diseases. These new studies have greatly advanced current understandings of enhancers. Yet at the same time they also created questions about the classical definition of enhancers in various aspects, from the functional roles of enhancers and relationships between them and their targets to the necessary and sufficient defining features (Figure 1, to be discussed in detail below). The once clearly defined concept of "enhancers" now appears to be fairly ambiguous.

In this era of high-throughput assays and genome editing, it will soon become possible to experimentally test the enhancer function of every region in the genome in an unbiased manner without the need for a candidate set of putative enhancers defined *a priori* [45–47]. Accordingly, the focus of enhancer studies will be switched from identifying the genomic locations of enhancers and testing the functional roles of a small subset of them, which has been an active area of research in the past few years, to interpreting and mining the high-throughput testing data. Depending on how enhancers are defined, the resulting sets of enhancers identified from such data can be drastically different.

In the followings we discuss issues related to enhancer definitions that are causing substantial differences among the enhancers reported in different studies, with our arguments supported by a survey of a representative set of 54 research articles about experimental and computational identification of enhancers and their targets (Table S1). The survey results show that in these studies the same term “enhancer” can actually refer to very different concepts, and sometimes a precise definition is not explicitly provided. We also illustrate the issues by comparing several major sets of human enhancers.

Based on these findings, we discuss the practical implications of the ambiguity and possible efforts for alleviating the situation, which would be fundamental for interpreting the results of single studies and comparing across multiple studies.

For the more general topics of enhancer properties and their experimental and computational identification methods, readers are referred to separate reviews [6,48–56].

Promoters initiate, enhancers enhance?

Classically, promoters and enhancers are considered separate classes of regulatory elements. This distinction has been substantiated by epigenomic studies, which identified different characteristic histone modifications for promoters and enhancers [57,58]. In this classical view, promoters provide the platform for transcription factors, co-factors and RNA polymerase to initiate transcription at a nearby transcription start site (TSS), whereas enhancers enhance transcription by coming in contact with promoters through looping, although the detailed mechanisms have not been completely mapped out. This dichotomy is also seen in our survey, in which 46% of the research articles explicitly exclude annotated promoters from the definition of enhancers.

However, this classical view has been challenged by a number of recent studies in several ways [4,58–62] (Figure 1a).

First, enhancers have been reported to produce short non-coding RNAs in one or both directions [4,63–66]. These enhancer RNAs (eRNAs) have even been considered hallmarks of active enhancers and used to quantify enhancer activities [18]. In our survey, 13% of the articles made use of data produced by protocols that could detect eRNAs, and it is becoming a popular feature for identifying enhancers.

Second, some promoters have been shown to enhance the expression of reporter genes when inserted near them [67,68]. In addition, using CRISPR-Cas9 to study the effects of a large number of mutations, disruption of some promoters were also found to affect expression of other genes that can be far away [46,69,70].

These findings blur the line between promoters and enhancers. Is it then possible to distinguish them by the transcripts they produce? In terms of transcript types, just like protein-coding genes, many functional non-coding genes also have their own promoters [71,72]; on the other hand, though not as commonly, enhancers can also transcribe messenger RNAs by acting as alternative promoters [30,73]. In terms of transcript properties, in contrast to mRNAs transcribed from promoters, eRNAs are generally shorter, unspliced and non-polyadenylated. However, exceptions do exist, such as some polyadenylated eRNAs with lengths that are even longer than typical mRNAs [74]. There is thus not a simple demarcation of the types of transcripts that can be produced at promoters and enhancers, and their difference lies more on the tendency of producing one type of transcripts or another.

In terms of directionality and stability, eRNAs can be unidirectional or bidirectional, which weakly correspond to the more stable and less stable transcripts, respectively [74]. In the same way, promoters have also been proposed to produce transcripts in both directions, which usually lead to upstream anti-sense RNAs (uaRNAs), or promoter upstream transcripts (PROMPTs) that are less stable, and a more stable transcript going the other direction. Both the length and stability of transcripts from the two directions depend on the presence of nearby U1 snRNP recognition sites and poly(A) signals (PASs) [75–77]. Directionality and stability, therefore, do not provide a simple black-and-white rule for classifying promoters and enhancers either.

Given all these similarities, whether a functional sequence element should be called a promoter or an enhancer could be just a matter of degree [78]. This view is best illustrated by the H3K4me3-to-H3K4me1 ratio, which is commonly used to distinguish between promoters and enhancers [79,80]. Just as this ratio can take on any non-negative value, “promoter” and “enhancer” could simply be the names given to the two extremes of a continuous spectrum of sequence elements having transcriptional initiating and enhancing abilities. It remains not clear though whether a sequence element can act as both promoter and enhancer simultaneously in the same cell type or even in the same single cell.

Distance-, direction- and orientation-independent?

Another core component of the classical definition of enhancers is that its distance, direction and orientation with respect to the target gene do not affect its capability of enhancing the gene's expression. Specifically, direction concerns whether the enhancer is upstream or downstream of the target gene, whereas orientation concerns the DNA strand that contains the enhancer sequence (Figure 1b).

There are many examples of the distance-independent nature of enhancers, but it should be noted that a lot of these studies involve reporter constructs in plasmids, which limit the maximum distance between the enhancer and the reporter gene to a few kilobases [81]. In native genomic contexts, indeed there are cases in which the distance between an enhancer and its target gene can be as far as 1Mbp [82–84], but the overall distribution of enhancer-target distance is skewed toward the low end with higher interaction frequency for shorter distance. Studies based on ChIA-PET, Hi-C and computational inference of enhancer-target interactions have estimated the median distance between an enhancer and the genes it regulates to be around 60-125kbp depending on the cell type [85,86]. For example, in IMR90 cells, around 25% of enhancer-target pairs are within 50kbp, while around 57% are 100kbp or more apart [86]. This distance distribution is strongly governed by the three-dimensional structure of chromosomes, where a large portion of enhancer-target interactions happen within topologically associating domains (TADs) or chromatin contact domains (CCDs) that are on the scale of hundreds of kilobase pairs to several megabase pairs in size [26,86–89]. All these data suggest that in genomes, there is a strong tendency for an enhancer to regulate genes that are close to it, and there are physical constraints due to the chromosome architecture that make it difficult for an enhancer to regulate genes very far away.

In artificial constructs, an enhancer can indeed enhance expression of a gene at various distances from it, yet the level of expression induction could still vary. Based on a variety of examples from the first discovery of enhancers to the latest research, with a displacement of hundreds of base pairs to several kilobase pairs, the level of induction can change for many folds [3,90–95].

Interestingly, in our survey we also found very different distance thresholds were used in different studies. At one extreme, some studies defined the potential target gene of an enhancer as the closest one or no more than 100kb apart. At another extreme, some studies allow potential target genes of an enhancer to be as far as

5Mb or even imposing no distance limits.

In terms of direction, most studies do agree that an enhancer should function no matter it is upstream or downstream of its target genes. In our survey, only two studies required a particular direction of the enhancers relative to the genes they regulate, but that is due to the design of the experimental protocol (of using the transcription of the enhancer itself in the transcript as an indicator of enhancer activity) rather than a believe that the enhancers would function better in that direction.

As for orientation, some studies have defined the stringent requirement that a DNA sequence can be considered an enhancer only if it can enhance expression no matter it is inverted or not [96–98]. However, many examples have shown that inverting an enhancer can in fact substantially alter the expression of the target gene [98–104]. Nonetheless, this issue seems to be not commonly considered in our surveyed articles, with none of them specifying the orientation explicitly.

Cis-acting?

The terms "*cis*" and "*trans*" are among biological concepts with the most erratic definitions. In the context of enhancers, they could mean whether the enhancer and target gene are on the same molecule (e.g., chromosome) [4,105–109], whether they are close to each other based on a certain genomic distance threshold [110], or whether the function of enhancer depends on its DNA sequence alone ("*cis*-regulatory element") but not its products ("*trans*-acting factors") [111–114] (Figure 1c).

The first definition, that an enhancer is *cis*-acting if it works only for target genes on the same molecule, originated from the SV40 enhancer. It increased transcription of the target gene by hundreds of folds when the enhancer was inserted together with the target gene in the same plasmid, but had no effect when they were inserted into separate molecules [3,49].

On the other hand, a strong evidence that enhancers can also regulate genes on another molecule, and thus work "in *trans*" by this first definition, comes from studies in Drosophila. It was found that enhancers can regulate genes on the other homologous chromosome by a phenomenon known as transvection [115–119]. Recent works suggest that transvection is fairly common in Drosophila [120], and it

appears to be a general property of all Drosophila enhancers [109].

Inter-chromosomal enhancer-target interactions can happen in species other than Drosophila, and, although relatively rare, between non-homologous chromosomes [121,122]. The general mechanisms of inter-molecular enhancer-target interactions remain unclear, but some evidence suggests that the formation of protein bridges may play a role [123].

In our survey, among the relevant articles, 95% of research articles required potential targets of an enhancer to reside on the same chromosome or DNA molecule as the enhancer. These studies thus assumed that enhancers act in cis according to this first definition. However, many of these studies likely made this decision mainly for the technical purpose of reducing false positives, at the expense of a (likely small) number of missed targets on other chromosomes.

The second definition of "*cis*" and "*trans*" based on genomic proximity is commonly used in the discussion of effects of single nucleotide variants (SNVs) and expression quantitative trait loci (eQTL) at a certain distance from the genes they affect [124–126]. As discussed above, some recent studies have estimated the distribution of genomic distance between an enhancer and its target gene in specific cell types. In order to determine whether enhancers act in *cis* then, what it takes is drawing a distance threshold and computing the fraction of such interactions involving enhancer-target pairs below the threshold.

Different values of this threshold have been used in different studies, such as 500kbp [18], 1Mbp [85,125,127] and 2Mbp [124,128], and these values appear to be arbitrarily picked. As mentioned above, in our survey we observed a variety of values used in our collection of articles. In order to find a suitable threshold, one could look at Hi-C contact maps and picked a threshold such that most DNA contacts happen between sites within this distance threshold [85,86,127,129,130]. However, this approach cannot solve the conceptual problem of finding a suitable distance threshold for "*cis*", since it presumes that most enhancers act in *cis*, and would thus lead to a circular argument if this threshold is then used to study whether enhancers are *cis*-acting in general. In addition, this approach still needs to decide on a threshold for defining "most DNA contacts", which could end up with another arbitrary choice such as 99%.

An extreme case of this second definition is that an enhancer is assumed to regulate

its closest gene. While simple and reasonably accurate given its simplicity, this approach could also lead to many missing targets. Indeed, in our survey only two articles have used this approach to determine enhancer targets.

The third definition is based on mechanism rather than genomic location. A *trans*-regulatory element produces diffusible factors (such as transcription factors, non-coding RNAs and signaling molecules) that regulate transcription of a gene by binding its *cis*-regulatory elements.

Traditionally, enhancers are described to interact with target promoters by forming chromatin loops [5], and thus they regulate target gene expression without necessarily involving any diffusible products. On the other hand, it has been discovered that the expression of eRNAs correlate positively with the expression of target genes [64,131]. Whether eRNAs play an active role in the transcriptional regulation process has become a question under debate, with three non-exclusive models [132]:

- The *trans* model, that eRNAs are free to move within the nucleus and play an active role in transcriptional regulation of promoters that could be far away. An example supporting this model is the Evf-2 noncoding RNA transcribed from an enhancer of Dlx-5/6, which complexes with the Dlx-2 protein and enhances its activity [81].
- The *cis-trans* model, that eRNAs are involved in mediating the interaction between the enhancer and target promoter [133].
- The *cis* model, that eRNAs are simply by-products when the transcription pre-initiation complex is brought close to the enhancer, and play no roles in regulating the target genes

In another classification [4], the *trans* and *cis-trans* models are grouped into a single class in which the eRNA has an active function, while the *cis* model is retained as a class with no functional roles for the eRNA. There is also a third class in which it is the transcription of eRNA, but not the eRNA itself, that is important for the regulatory process.

The lack of knowledge about eRNA functions is also reflected in our survey, with only seven articles trying to discuss this topic.

Before the functions of eRNAs in transcriptional regulation (if any) become clear, no

conclusions can be made as to whether enhancers are largely *cis*-acting based on this third definition of "*cis*" and "*trans*".

In general, few studies have explicitly considered all the above three definitions of "*cis*" and "*trans*" when studying enhancers, but a number of reviews on enhancers have already explicitly pointed out that enhancers can act both in *cis* and in *trans*, based on certain definitions of these terms [49,134,135]. A study also no longer mentions "*cis*-acting" in the definition of enhancers, but describes them in a general way as "regulatory elements that increase the transcriptional output of target genes" [136].

Necessary or sufficient?

The classical definition of enhancers does not explicitly specify whether a sequence element should be necessary or sufficient for enhancing the expression of a gene in order to be called an enhancer. It also does not specify whether every nucleotide in the enhancer sequence is necessary (Figure 1d).

Considering an enhancer as a whole, one obvious reason that necessity should not be considered in defining enhancers is the possibility for a gene to be regulated by multiple enhancers. It has been estimated, based on 3C-based methods and computational predictions, that each gene is regulated by on average 2-5 enhancers in each cell/tissue type, depending on the exact cell/tissue type and whether only genes with at least one regulating enhancer are considered [85,86]. For some genes, when the main enhancer is absent, other "shadow enhancers" can take up the role [137]. Recent studies indicate that shadow enhancers may be quite prevalent, at least in the Drosophila genome [138]. To complicate things further, enhancer redundancy may change with the context. For instance, an enhancer having redundancy provided by another enhancer under normal conditions could become essential under more stressful conditions [139,140]. In general, the fact that an enhancer is not necessary for a target genes' expression due to the presence of other enhancers does not naturally affect its status as an enhancer.

If enhancers are to be defined based on their sufficiency instead, one would consider a sequence element an enhancer if there is a detectable amount of induction of the target gene's expression when the enhancer becomes present/active. This is the principle behind reporter assays, that the expression of the target gene with only a weak promoter is compared to the expression when the enhancer is present. There

is no standard as to the amount of induction required for the tested sequence to be considered an active enhancer, but in general defining whole enhancers by their sufficiency is commonly accepted.

It becomes trickier if we consider individual nucleotides within an enhancer. When a sequence is considered an enhancer, does it mean every nucleotide is necessary for the enhancer function, or some nucleotides can be dispensable as long as the whole sequence is sufficient for enhancing target gene expression?

Since an enhancer sufficient for enhancing the expression of a gene may contain regions not necessary for its function, various studies have attempted to define minimal enhancers by progressively deleting DNA sequences from both ends of an enhancer until its enhancing ability is largely abolished [97,98,141–147]. These studies implicitly define minimal enhancers as a sequence element that is sufficient as a whole, and every nucleotide is necessary.

Interestingly, some authors went further and defined an enhancer by the necessity of the nucleotides alone. For example, Smyth et al. (2008) identified a 20bp region that is necessary but not sufficient for receptor cone photoreceptor-specific expression, and called it an enhancer.

As a recurrent theme of this review, while sufficiency and necessity are concepts of Boolean logic, in reality the expression level of a gene is not binary. This is well exemplified by an early study of an enhancer of the type II collagen gene [148]. A sequence of 1.5kb was identified to enhance the expression of this gene. Taking the minimal enhancer approach, a 100bp sub-sequence was found to be able to drive the collagen gene expression at a similar level. However, when 6 additional nucleotides were deleted from the 5'end of this 100bp sequence, expression dropped to 68%. When 15 nucleotides were deleted from the 5'end instead, expression dropped to 10%. Similarly, when 11 nucleotides were deleted from the 3'end, expression dropped to 78%. While the 100bp sequence was clearly sufficient for the enhancer activity, whether the 94, 85 and 89bp truncated sequences should be considered sufficient is again a matter of choosing an expression threshold of the collagen gene. This type of gradual reduction of enhancer activity when trimming off more and more nucleotides can also be found in other studies [12,149].

One may be tempted to take a simple approach to consider an enhancer sufficient only if it can drive the full expression of the target gene. The full expression level is

usually defined based on a certain reference situation, such as the original genomic context. However, there could be silencers and insulators in the original sequence, the deletion of which can lead to an expression level of the target gene even higher than the natural context, thereby contradicting the definition of “full” expression [99,142]. Similarly, when multiple enhancers can regulate the same gene, it is unclear whether full expression should be defined based on the enhancers that actively regulate the gene in the natural context, or when all of them are made active artificially. Furthermore, the exact sequence of an enhancer may also affect the degree of target gene induction. For example, when the natural spacing between GATA1 and ETS1 binding sites in the Otx-a enhancer is increased, the enhancer activity can be increased as well [150]. These and other factors make it difficult to define the full expression of a gene.

In our survey, we found that most studies defined enhancer lengths based on practical considerations, such as size of enzyme-digested DNA fragments or length of ChIP-seq signal peaks, rather than functional necessity. We did observe a growing trend of large-scale random perturbations of enhancers, which provide information about the effects of individual nucleotides and help define the necessary regions of enhancers as discussed below.

By effect, features or mechanism?

A systematic way to test enhancer function is to perturb a potential enhancer sequence and observe the resulting change of target gene expression. For example, saturation mutagenesis coupled with massively parallel reporter assay has enabled studying the effects of mutated sequence elements on reporter genes [54]. With the invention of genome editing methods based on CRISPR-Cas9, it is now also possible to perturb specific sequences genetically [30–37] or epigenetically [31,38–44]. In these experiments, if the expression level of a gene after the perturbation of a sequence element is reduced, the sequence element is a potential regulator of the gene. Some of these elements have been found to lack typical chromatin and epigenetic features of enhancers [46,70]. Consequently, they were named unmarked regulatory elements (UREs) in some studies [46].

If we take the perturbed sequence as reference, having the unperturbed UREs can increase the target gene expression, and thus they can be called enhancers. One may further require that these UREs to be able to drive reporter gene expression in reporter assays before they can be called enhancers.

Yet the conundrum is that UREs do not have typical features of enhancers. Is it necessary for a sequence element to contain some features indicative of enhancers in order to be qualified as an enhancer? To answer this question, first there should be a common set of features used for defining enhancers. Many different feature sets have previously been used, such as H3K4me1, H3K27ac, P300 binding and eRNA [18,149,151–153]. Extensive comparisons of these features have revealed that different features have different strengths and weaknesses [154,155]. While the debate of the best enhancer-defining feature set continues, it is clear that no single feature can identify enhancers without false positives and false negatives. In our survey, 98% of the relevant articles used some features to define enhancers, but the features used differed substantially from study to study. There are also features such as H3K64ac and H3K122ac recently proposed to define a new class of enhancers [156]. Whether UREs can be considered enhancers could therefore evolve over time as more features of enhancers are discovered.

The fact that disruption of a sequence element reduces target gene expression could also be due to other factors, such as the change of the local chromatin structure. Reduction of target gene expression alone is therefore insufficient for concluding that the sequence element plays enhancer role functionally. In the literature, enhancers are usually described to function by binding transcription factors and co-activators, forming a loop to get in touch with the target promoter [6,130]. Should a sequence element be called an enhancer only if it satisfies these criteria [81]? With an incomplete list of proteins that bind enhancers and the lack of DNA three-dimensional contact information, currently it is difficult to define enhancers based on these mechanistic criteria. As a result, most “enhancers” reported in the literature were based on either their outcomes or features instead of their proven mechanisms (Figure 1e). In our survey, among the articles that tried to study the mechanisms of enhancers, the majority determined physical contacts of promoters and enhancers, which should be considered relevant but not a direct proof of the mechanistic functions of the enhancers.

Illustration of the issues by comparing several major enhancer sets

To further illustrate the issues discussed above, we compared several major sets of human enhancers to see whether they have differences related to enhancer definitions:

1. Roadmap+ENCODE [157]: Enhancers inferred by the Roadmap Epigenomics

Consortium for 127 human cell and tissue samples (including some cell lines from the Encyclopedia of DNA Elements, ENCODE) based on histone modifications and other chromatin features

2. FANTOM5 [18]: Enhancers defined by CAGE (Capped Analysis of Gene Expression) signals that exhibit eRNA-like patterns from around 1,800 human cell and tissue samples
3. VISTA [158]: Human enhancers identified from various methods tested *in vivo* using transgenic mouse assays
4. TCGA [159]: A subset of FANTOM5 enhancers with expression signals in around 9,000 human tumors based on RNA sequencing (RNA-seq)

Before comparing the genomic locations of the enhancers in these data sets, we already observed various significant differences among them, including the average length (from 276bp to 2,043bp), total number (from less than 1,000 to more than 9.3 million) and saturation (Figures 2a and 2b) of the enhancers. In particular, the FANTOM5 enhancers have saturated after including around 150 samples, but the Roadmap+ENCODE ones are not yet saturated after including all 127 samples. If the two sets of enhancers are directly compared, the differences observed could be due to either the incompleteness of the Roadmap+ENCODE set or intrinsic differences of the enhancers from the two sources.

Therefore, we compared the four data sets in three different ways with three corresponding goals. In the first comparison, we divided the whole genome into 200bp bins and considered each of them as an enhancer bin if it overlapped with an enhancer by at least 100bp, which allowed us to easily determine the intersections of the four sets. From the results (Figure 2c), the different sets of enhancer bins intersected poorly, with only 73 of them commonly shared by all four sets. This is due to a combination of reasons, including the different cell and tissue types involved in defining the different sets, their different types of samples, their ways of defining enhancers (by features or by reporter activities), as well as other technical differences such as how the length of each enhancer was determined (based on feature signals or trimmed to the same length). We also acknowledge that there are sub-structures among the data sets, in that the TCGA enhancers are a subset of the FANTOM5 enhancers, while many VISTA enhancers were originally selected based on chromatin features similar to the features used by Roadmap+ENCODE. Therefore, the four data sets tend to form two clusters that are less similar from each other.

Another way to interpret the results is by the ratio of overlap. For example, around

88% of FANTOM5 enhancer bins were also in Roadmap+ENCODE, while only around 1% of Roadmap+ENCODE enhancer bins were also in FANTOM5. Given that Roadmap+ENCODE contained almost 100 times more enhancers than FANTOM5, it is surprising to see that 12% of FANTOM5 enhancer bins were still not covered by Roadmap+ENCODE. One potential reason could be that FANTOM5 contained a lot more samples, and thus some cell- and tissue-type specific enhancers could not be covered by Roadmap+ENCODE. However, even when we included only 127 random samples from FANTOM5, there were still 12% of enhancer bins not covered by Roadmap+ENCODE (Figure 2d), showing that these two sets were different also in other additional ways. Since FANTOM5 and VISTA also contained mouse enhancers, we repeated the above analysis and found that the two resulting sets of enhancer bins also intersected poorly (Figure 2e).

As mentioned above, a potential issue of the above comparisons was the different lengths of enhancers from the different sets, such that longer enhancers would occupy more bins, and it could happen that only some of them intersected with the enhancer bins from another data set, even if both sets contained a similar enhancer. Therefore, in the second setting, we compared the enhancers directly rather than enhancer bins, and varied the required fraction of overlapping bases for two enhancers to be considered the same. An exception was made here for the Roadmap+ENCODE enhancers, which we still used 200bp bins since its enhancers from different samples do not align, unlike the FANTOM5 enhancers. We performed such comparisons for every pair of data sets. From the results (Figure 3), only in two cases was one set of enhancers completely covered by another set, namely VISTA enhancers covered by Roadmap+ENCODE (Figure 3d) and TCGA enhancers covered by FANTOM5 (Figure 3e). Both cases were due to technical rather than biological reasons as explained above. In all other cases, the overlap ratios were not very high. For example, only around 80% of the FANTOM5 enhancers could be covered by Roadmap+ENCODE even when two enhancers would be considered overlapping when they had only minimal overlap (Figure 3b, required fraction of overlapping bases close to 0%), suggesting that differences in enhancer lengths could not explain the 12% FANTOM5 enhancer bins not covered by Roadmap+ENCODE in the first comparison setting.

Finally, to deal with the issue that the different enhancer sets included different biological samples, we compared the enhancers in four ENCODE cell lines included in both the FANTOM5 and Roadmap+ENCODE data sets. Using the same comparison strategy as in the second setting above, but considering full-length

Roadmap+ENCODE enhancers rather than 200bp bins, we again observed that the overlap ratios were low (Figure 4), showing that discrepancies in biological samples also cannot explain the differences among the enhancer data sets.

Overall, this comparison of the four enhancer sets reveal that they are very different and there are many factors behind their differences. Some of these factors touch on the issues about enhancer definitions discussed above, such as whether enhancers are defined by features (CAGE/eRNAs or histone marks and DNA accessibility) or by effects (reporter activities), whether distance and orientation to target gene are involved (when designing the reporter construct), and how the exact span of the enhancer region is determined (arbitrarily or based on patterns of feature signals).

Practical implications and possible actions

Considering all the aspects of enhancer definition discussed above, and the fact that in our surveyed articles enhancers have been defined in very different ways, we argue that instead of trying to give a single universal definition of enhancers, it is more useful to define enhancers in a case-by-case manner according to the practical purpose. Here we illustrate this idea by discussing several key contemporary problems related to enhancers.

Benchmarking the performance of enhancer identification methods

The power of benchmarking computational and experimental methods based on common data sets, standardized procedures and multi-faceted evaluations has been well-demonstrated by various contests covering topics from low-level tasks such as sequence assembly and transcript quantification to high-level tasks such as prediction of structures, functions and interactions [160–165]. While many methods have been proposed for identifying enhancers, there has not been an analogous large-scale open contest for benchmarking these methods [166].

There are two major ways for setting up such a contest, namely pre-creating a “gold-standard” set of positive and negative enhancers and asking the competing methods to distinguish between them, or post-validating the candidate enhancers identified by the contesters experimentally. In both cases, the experimental details involved play a major role in defining sequences that should be considered an enhancer. First, reporter assays test sequences that are sufficient for driving reporter gene expression, while perturbation experiments test sequences that are necessary

for maintaining target gene expression, leading to different objectives. Second, the length requirements of the sequences to be tested, whether they will be automatically extended to a minimum length, as well as the distance, direction and orientation to the target gene can all affect the prediction strategy. Third, since enhancer activity is affected by the chromatin environment and the availability of relevant transcription factors, if the experimental method depends on these factors, it is necessary to provide some relevant information, for example by means of supplementary ATAC-seq and RNA-seq data of the testing system.

Enhancer annotation

There are various sets of comprehensive annotations for genes, with a reasonably high level of consistency between them, at least for protein-coding genes [167]. Correspondingly, by defining promoters as a (albeit arbitrary) small region around TSSs, they are also fairly well defined. In contrast, enhancer annotations are far from having the same level of convergence, as demonstrated in the comparisons presented in the introduction.

Going forward toward a more unified set of enhancers, there are various lessons that can be learned from gene annotations, which naturally land on definitions of enhancers. First, gene annotations aim at including the full set of genes and transcripts. A gene is included as long as it is expressed in at least one cell/tissue type or condition. In the same way, an enhancer annotation set should include all sequence elements that have enhancing capability in any context, regardless of the exact definition. Second, just as genes are annotated by the different sub-elements of it (exons, introns, etc.) and at different levels (elements related to transcription and elements related to translation), enhancers can also be annotated by different sub-elements based on their functions (such as protein binding locations), features (such as location of H3K27ac peak) and outcomes (such as parts that can drive the target gene up to certain percentage of a reference expression level). Third, some gene annotations provide confidence levels based on experimental evidence, manual verification and computational inference [167]. Enhancer annotations can as well be associated with confidence levels based on such information. This three-level hierarchy of information allows users of the enhancer annotations to pick the most suitable enhancer definition and correspondingly the boundaries of each enhancer based on their need.

Identifying target genes of enhancers

Since the target genes of an enhancer can be upstream or downstream of it and at various distances, determining enhancer targets is a non-trivial task and many methods have been proposed [19,20,29,21–28]. Having a clear definition of enhancers helps clarify the scope of this task and guide the design of the methods.

Most existing methods define a maximum distance between an enhancer and its potential target genes, due to physical, computational and statistical reasons. If enhancers act in *cis* alone, setting this maximum distance to the typical size of a TAD should be sufficient to cover a large portion of enhancer targets. On the other hand, if enhancers can act in *trans* by having functional roles of eRNAs over a long genomic distance, this distance threshold should be abandoned and it could be useful to incorporate new types of information into the target identification methods, such as protein-eRNA interactions [168].

As discussed above, the boundaries of an enhancer are hard to define, depending on the sufficiency/necessity requirement and level of target gene expression that it needs to drive. Enhancer identification methods based on activity signals could lead to enhancers that are slightly shifted in different samples, making it difficult to define a unified set of enhancers. In addition, whether a large genomic region with broad enhancer feature signals should be considered a single enhancer unit (such as a super enhancer [169,170]) or multiple separate enhancers also affects the target identification methods. A general way to deal with these issues is to divide up such broad regions into small enhancer units and allow them to exert joint effects on common targets [29].

Estimating functional effects of non-coding genetic variants

An important application of enhancer annotation and enhancer target gene sets is to estimate changes of gene expression levels due to non-coding genetic variants that hit enhancer regions [171,172]. Existing methods mostly look for simple overlaps between the variants and annotated enhancers, or specific protein binding motifs or ChIP-seq signals within them. Having a detailed sufficiency/necessity map of individual nucleotides for enhancer function could greatly improve the precision of these methods.

A straightforward approach to predicting genes affected by a non-coding variant is to associate the variant with genes nearby, such as the closest genes, genes within a

certain genomic distance, or genes within the same linkage disequilibrium block [173]. Although enhancers may not regulate genes within the immediate vicinity, this simple approach is still fairly popular [174]. Advancements in enhancer target identification methods, taking into account the operational mechanisms of enhancers, will help predict the affected genes and accordingly better prioritize genes for validation and functional experiments.

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Key Points

- The traditional definition of transcriptional enhancers needs to be revisited due to various recent new findings.
- In the literature, many different definitions of enhancers are used, making it difficult to compare the results in different studies.
- It is important for the research community to define clear reporting guidelines regarding the definition of enhancers adopted.

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Figure Legends

Figure 1. Different aspects that affect the definition of transcriptional enhancers. (a) In the classical view, a promoter initiates transcription while an enhancer enhances the expression level. However, it has been shown that enhancers can also initiate transcription, and promoters also exhibit enhancing activities. (b) The expression level of the target gene could be affected by the distance and direction of the enhancer with respect to the gene, and its orientation. (c) Three different definitions of *cis* and *trans*, respectively based on whether the enhancer and target gene are on the same molecule, whether the enhancer is close to the target gene, and whether the enhancer itself but not its diffusible products is needed for the enhancer function. (d) A sequence element is usually defined as an enhancer if it is sufficient to drive target gene expression, even if it is not necessary due to alternative enhancers. On the other hand, necessity of individual bases can be defined based on the loss of target gene expression upon removing the bases. (e) Enhancers are commonly defined based on its features such as H3K4me1 and H3K27ac and/or by its observable effects on target gene expression, although ideally the mechanism should be involved in the definition.

Figure 2. Comparisons of the four enhancer sets based on fixed-sized bins. The whole genome was divided into consecutive, non-overlapping 200bp bins. For each enhancer set, a bin was defined as an enhancer bin if it overlapped with an enhancer from this set by at least 100bp. (a,b) Saturation plots of the FANTOM5 (a) and Roadmap+ENCODE (b) human enhancer bins, where the y-axis represents the fraction of all enhancer bins that can be covered by a random subset of the samples. For each subset size, 100 random subsets of samples were drawn to form a distribution. (c) A Venn diagram of the four sets of human enhancer bins. (d) The fraction of FANTOM5 human enhancer bins covered by Roadmap+ENCODE based on all FANTOM5 samples or subsets of 127 random samples, with the distribution of the latter formed by 100 random subsets. (e) The intersection of FANTOM5 and VISTA mouse enhancer bins.

Figure 3. Pair-wise comparisons of the four enhancer sets at various fractions of overlapping bases for two enhancers to be considered the same. Each panel involves a pair of enhancer data sets. Each curve in a panel shows the fraction of enhancers from a particular set (the “subject enhancers”) that are also contained in the other enhancer set, with the fraction of overlapping bases computed using the enhancer length of one of the two sets chosen (the “length normalizer”) as the denominator.

For example, when the subject enhancers are from FANTOM5 and the length normalizer is the Roadmap+ENCODE enhancers, a point on the curve with an x-coordinate of *x* indicates the ratio of FANTOM5 enhancers that are also contained in Roadmap+ENCODE, where a FANTOM5 enhancer is considered to be contained in Roadmap+ENCODE if there is an enhancer from Roadmap+ENCODE of length *l* that share *c* common bases with the FANTOM5 enhancer with $c/l \geq x$.

Figure 4. Pair-wise comparisons of the FANTOM5 and Roadmap+ENCODE enhancer sets at various fractions of overlapping bases for two enhancers to be considered the same, based on only enhancers from the same cell lines. Each panel involves only the enhancers from a particular cell line contained in both the FANTOM5 and Roadmap+ENCODE data sets. The interpretation of the curves is the same as in Figure 3.

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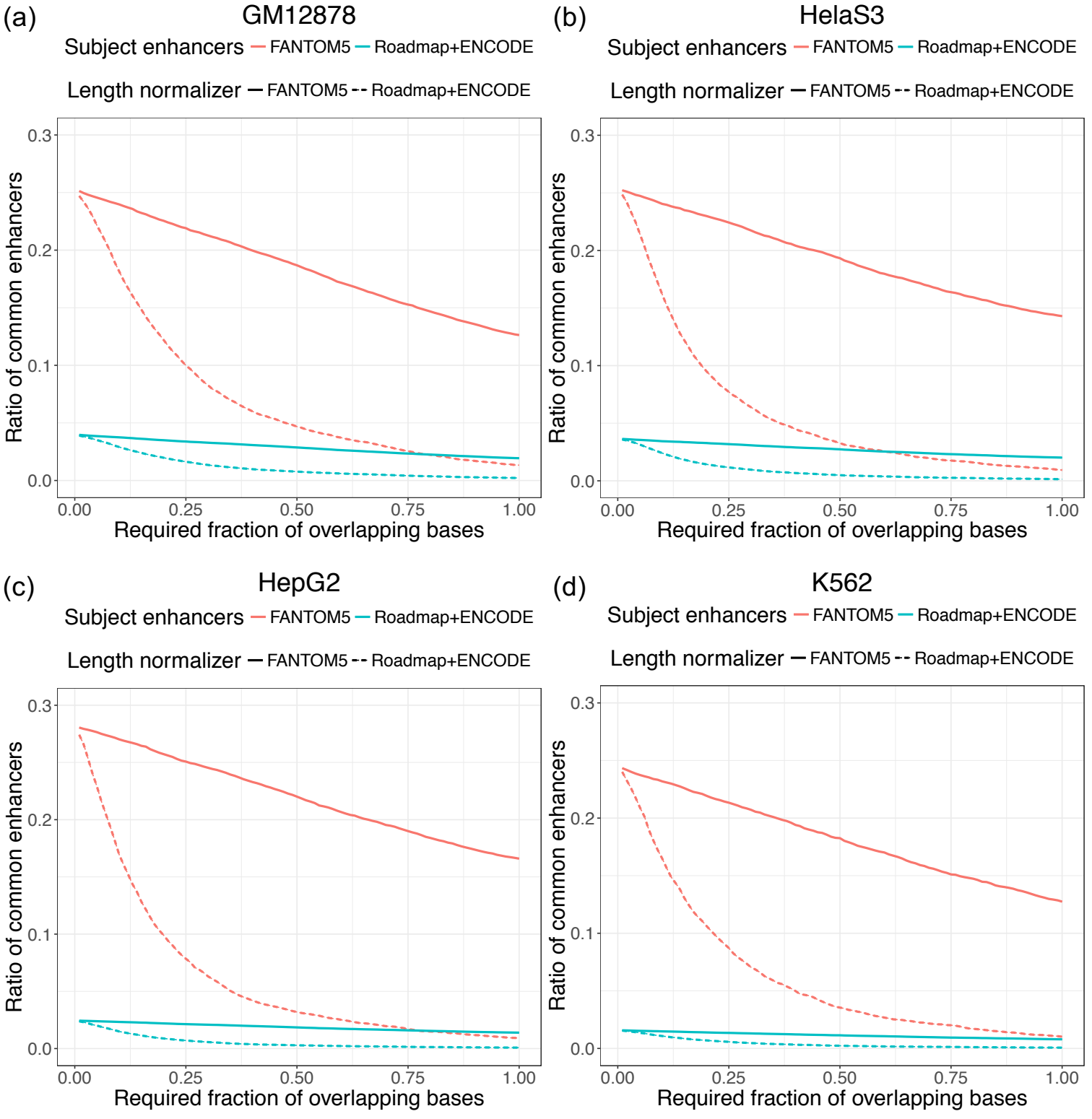
(a)
GM12878
Subject enhancers FANTOM5 Roadmap+ENCODE
Length normalizer FANTOM5 Roadmap+ENCODE
Ratio of common enhancers
0.0
0.1
0.2
0.3
0.00
0.25
0.50
0.75
1.00
Required fraction of overlapping bases
(b)
HelaS3
Subject enhancers FANTOM5 Roadmap+ENCODE
Length normalizer FANTOM5 Roadmap+ENCODE
Ratio of common enhancers
Required fraction of overlapping bases
(c)
HepG2
Subject enhancers FANTOM5 Roadmap+ENCODE
Length normalizer FANTOM5 Roadmap+ENCODE
Ratio of common enhancers
Required fraction of overlapping bases
(d)
K562
Subject enhancers FANTOM5 Roadmap+ENCODE
Length normalizer FANTOM5 Roadmap+ENCODE
Ratio of common enhancers
Required fraction of overlapping bases