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Genome organization and long-range regulation of gene expression by enhancers

Andrea Smallwood[1] and **Bing Ren**[1,2]

[1]Ludwig Institute for Cancer Research, United States

[2]University of California San Diego, School of Medicine, Department of Cellular and Molecular Medicine, Institute of Genomic Medicine and Moores Cancer Center, 9500 Gilman Drive, La Jolla, CA 92093-0653, United States

Abstract

It is now well accepted that cell-type specific gene regulation is under the purview of enhancers. Great strides have been made recently to characterize and identify enhancers both genetically and epigenetically for multiple cell types and species, but efforts have just begun to link enhancers to their target promoters. Mapping these interactions and understanding how the 3D landscape of the genome constrains such interactions is fundamental to our understanding of mammalian gene regulation. Here, we review recent progress in mapping long-range regulatory interactions in mammalian genomes, focusing on transcriptional enhancers and chromatin organization principles.

Introduction

Disease associated SNPs are predominantly found at enhancers, yet the precise outcome of such mutations is unclear largely due to our incomplete knowledge of their specific regulatory targets *in vivo* [1•]. An enhancer is operationally defined as a portion of DNA that can activate transcription from a target promoter in an orientation and location independent manner [2,3]. Enhancers contain consensus binding motifs for both common and cell-type specific transcription factors (TFs) and are therefore thought to be hubs for TF binding [4]. Given this limited definition it has proven difficult to identify enhancers on a genome-wide scale. However in recent years, with the advent of next generation sequencing techniques, our ability to identify new enhancers has dramatically increased [5]. Early studies found that distal p300 binding sites, identified using chromatin immunoprecipitation followed by high throughput sequencing (ChIP-seq), function as tissue-specific enhancers [6]. These sequences were found to be enriched in H3K4me1 but depleted for H3K4me3, whereas promoters were enriched for H3K4me3 [7]. This epigenetic signature has been used to predict novel enhancers across multiple cells types including ESCs and a variety of differentiation lineages in multiple species including mouse and humans [6,8–12,13•]. The epigenetic definition of an enhancer has more recently been expanded with the discovery that so-called ‘active’ enhancers are also marked with H3K27ac in ESCs, whereas ‘poised’

Corresponding author: Ren, Bing (biren@ucsd.edu).

enhancers can also be marked by H3K27me3 [14,15]. Binding of the histone acetyltransferases (HATs) p300 and ATAC are also enriched at enhancers [6,7,16], along with ATP dependent chromatin remodelers such as CHD7 and Brg1 [15,17]. In addition to ChIP-based approaches, distal DNaseI hypersensitivity sites have also been utilized successfully for enhancer predictions, leading to the identification of millions putative enhancers in the human genome [18••,19], representing a great advance towards the understanding of gene regulatory networks in mammals. Despite these rapid progresses, it remains a great challenge to link the enhancers to their regulatory target genes. Identification of such linkages is not straightforward as enhancers are predominantly located some distance from their target genes. Characterization and identification of enhancers has been extensively described elsewhere [20,21] and is therefore not the focus of this review. Here, we instead will review the identification of enhancer–promoter interactions and the role of genome architecture in the control of gene expression.

How do enhancers contribute to gene regulation from a distance?

It was originally thought that TF binding at enhancers affected transcriptional output by directly causing recruitment of RNA polymerase II and assembly of a full preinitiation complex [22]. However, for this to be true, the distance between an enhancer and its target promoter must be drastically decreased. It is now generally believed that enhancers and promoters are brought together via DNA looping. The earliest studies suggesting looping as a method of gene regulation were performed in *Escherichia coli*; including repression of the *AraC* gene and activation at a distance of *NtrC* [23]. Another early study, utilizing a proximity ligation technique, observed that activation of the PRL gene by ER involves looping of a distal enhancer to the proximal promoter [24]. Subsequent studies with artificial enhancer–promoter arrays have also demonstrated that TF binding sites at enhancers cause looping of DNA and directly contact TF binding sites at the promoter [25]. More recently, it was observed that TF requirement could be by-passed by artificially looping enhancers and promoters together. In this system the presence of TF co-factors was sufficient to allow for transcription initiation [26•]. The model that enhancers illicit a positive effect on transcription by forming these loops and allowing physical interactions with promoters has been tested and visualized by FISH and the PCR based chromosome conformation capture assay (3C) [27]. 3D interactions in α-globin and β-globin loci have been exhaustively studied by many groups utilizing 3C technologies. Transcription factor KO abrogating β-globin gene transcription also abolishes promoter and the β-globin locus control region (LCR) interaction [28,29]. Additionally, the β-globin locus is found looped out of its surrounding chromosomal region specifically in erythroid cells. This rearrangement is dependent upon the LCR and occurs before gene activation, suggesting that looping is not just a result of gene activity but a cause of it [30]. In fact, ectopic integration of the LCR can cause relocation of neighboring genes to more euchromatic or active regions of the nucleus [31]. This wealth of evidence suggests that enhancers are playing an active role in chromatin arrangements to affect gene expression. Furthermore, studies of enhancer promoter interactions have demonstrated that gene activity is positively correlated with the number of interacting enhancers [32]. Nevertheless, a major bottleneck in the field still remains; how do we identify the functional targets of enhancers in any given cell or tissue type? This is all

the more important given the sheer number of enhancer elements predicted thus far as compared to the number of promoters [33•].

Methods to identify targets of enhancers

Traditionally, enhancers were thought to act on their nearest genes along the linear DNA. However, genetic studies in mouse models or humans showed that this is not always the case. For instance, analysis of the Ssq (Sasquatch) mouse mutation demonstrated that disruption of an enhancer, found 1 Mb away in an intron of the *Lmbr1* gene, is responsible for misexpression of the *Shh* gene and polydactyl phenotype [34]. 3C has proven to be a powerful tool for dissection of the relationship between specific key enhancers and target promoters within gene loci. However, it is limited by the need for prior knowledge of possible interactions in order to design primers to the specific regions to be tested. 4C and 5C couple 3C with next generation sequencing techniques, allowing for a broader ‘one to all’ (4C) or ‘many to many’ (5C) view of chromosome contact frequencies in the genome (reviewed in [35,36]). 4C was utilized to identify β-globin interactions dependent on expression status of the gene [37]. If inactive the gene contacted other repressed regions whereas an active β-globin gene localized with other active regions supporting previous results suggesting large-scale rearrangements of this region. In addition, this technique has been applied to study the chromatin topologies occurring with X-chromosome inactivation and Polycomb group repression of genes [35,38]. 4C provides genome-wide interrogation of interactions but only from a single gene locus bait point. However, 4C lacks data complexity and the resolution necessary to detect short-range interactions due to the choice of restriction enzymes currently used to fragment the genome. 5C maps have been generated for several cell types and predicted interactions correlate well with gene expression analysis, but this technology still lacks the coverage necessary for a whole genome view [39,40••]. Collectively, such studies have demonstrated that active regions are frequently looped into compact topologies [35]. Presumably these structures increase the potential of enhancer promoter interactions. A related technique, ChIA-PET, couples ChIP with 3C conformation capture analysis [41]. In this way one can focus on interactions occurring between specific regulatory elements mediated through a transcription factor complex or histone proteins. To date, interactome maps of ER-α, CTCF, H3K4me2, and RNA polymerase II associated interactions have been produced [32,41,42,43•]. For all proteins studied thus far ChIA-PET appears to favor recovery of more local contacts that lie in close physical distance along the same chromosome [35]. Additionally, this technique only reveals a correlative relationship between factor binding and looping but not necessarily a causative one. Hi-C, a 3C like technique, allows for the most comprehensive coverage of genome interactions thus far [44•]. Resolution is still limiting factor for Hi-C techniques, mostly due to the sequencing depth required to detect interactions but also the choice of restriction enzyme and frequency of cutting as seen for 4C (Figure 1). The pros and cons of each technique, for interrogating chromosomal interactions and 3D topologies within the genome, have been described in detail in recent reviews [35,36].

Chromosome conformation capture techniques have furthered our conceptual understanding of genome organization but resolution is still a limiting factor. Therefore, recent studies have utilized computational tools to integrate multiple data sets such as ChIP-seq, DNaseI-seq,

and RNA-seq as a method for predicting enhancer and promoter interactions. Ernst *et al.* employed logistic regression to integrate gene expression levels, enhancer and promoter ChIP-signals, and sequence motif enrichment data sets across nine different cell types [45]. The authors then calculated linkage scores for enhancers and potential target promoters. However, this approach factored in a distance-based bias which is inconsistent with the idea that genes may be regulated by enhancers across great distances [46]. A similar study first calculated signal intensity for enhancers and promoters, using H3K4me1 and RNA Pol II ChIP-seq respectively, across 19 distinct mouse tissue and cell lines [13•]. Enhancer–promoter linkage, for each possible pair in the genome, was analyzed using Spearman correlation coefficiency of signal intensity. It was found that co-regulated elements clustered into enhancer–promoter units or EPUs. Interestingly, EPUs averaged a ratio of 5.67 enhancers per promoter, which correlates well with current hypotheses. Moreover, comparison of Hi-C and EPU analysis demonstrated that physical demarcation of the genome correlated well with functional clustering. Multiple predicted enhancer–promoter interactions were subsequently validated by 3C analysis. Another method to link enhancers to functionally relevant target promoters utilized DHSs mapped in over 79 diverse cell types [18••]. In this study, the patterning of distal DHSs was compared to all promoter associated DHSs within ±500 kb. This allowed generation of a map of highly correlated enhancer promoter connections. The proposed interactions were validated using multiple 5C experiments including ‘promoter-to-all’ analysis of 1% of the human genome [40••]. The data set also correlated well with previously published interactions elucidated by RNA Pol II ChIA-PET in K562 cells [43•]. The above correlation-based approaches are all based on the assumption that an enhancer and its target gene share similar activity profiles across many tissues. However, there are two potential problems with this assumption. First, two elements may share similar profiles because they are regulated by a common transcription factor, not necessarily because one is a target of the other; second, a promoter driven by different sets of enhancers in different tissues may not share similar activity profiles with its enhancers. So the validity of this approach will need to be tested by further experiments, such as evidence of long-range looping. Nevertheless, the initial results so far appear to nicely capture targeting relationships between tissue-specific promoters and enhancers.

Topological domains

Hi-C provides us with a hierarchical view of chromosome structure on a genome-wide scale. Initial Hi-C analysis of human cells at a low resolution demonstrated that the genome is organized into active and repressed chromatin in separate multi-megabase-sized genomic compartments [44•]. Recent experiments have analyzed the genome organization in multiple cell types and across multiple species at a higher resolution [47••,48••]. These studies demonstrate that the genome is organized into thousands of megabase-sized, locally interacting regions termed ‘topological domains’ or ‘Topologically Associating Domains’ (TAD) [47••,48••]. Each domain contains an average of tens of genes and hundreds of enhancers. It is not hard to imagine that these self-interaction domains might constrain looping interactions between enhancers and promoters and set the boundaries of coordinated gene regulation. Consistent with this hypothesis, the Shh enhancer discussed above is located 1 Mb away from the *Shh* gene, but it falls within the same topological domain

(Figure 2). This domain architecture and enhancer–promoter relationship appears to be conserved in different species and cell types. In B cells, genes found within the same defined block are coordinately regulated more frequently than genes residing in different blocks [49]. It has also been demonstrated that *Xist* and *Tsix*, whose expression is anti-correlated, are separated into adjacent domains along with their known regulators [48••]. The same study compared TADs with gene expression changes during mESC differentiation and showed that correlation between genes upregulated or downregulated is higher within domains than between them. Disruption of a boundary between two TADs resulted in misregulation of transcription over a large range coupled with distinct changes in chromosome topology [48••]. These data demonstrate physical and functional clustering regulatory elements into topologically defined domains. These domains also function to demarcate active and repressed regions of the genome and contain the spread of H3K9me3 after differentiation [47••,50•]. Interestingly, topological domains appear to be largely invariant across cell types and surprisingly conserved between human and mouse, suggesting that physical partitioning of the genome is a fundamental principle of genome organization. Upon closer examination, cell-type specific differences have been observed [47••,48••,49]. However, these differences appear to mostly be changes in how chromatin is organized or folded within topological domains. For instance, RNA and DNA FISH experiments show that self-association of TADs is decreased in inactive versus active X-chromosomes in mouse cells suggesting a more random structure of the inactive X-chromosome [48••]. Importantly, dynamic chromatin interactions revealed by Hi-C analyses of mouse ESC and cortex were found to be enriched for cell-type specific expressed genes, and nearly entirely constrained within topological domains [47••].

Mechanisms of topological domain formation

The strong conservation of topological domains in different cell types and through evolution suggests sequence elements as a driving force behind the physical partitioning. Indeed, comparison of Hi-C and ChIP-seq data in IMR90 and mES cells demonstrated that CTCF binding sites are enriched at topological domain boundaries. For instance, a Hi-C domain boundary corresponds to a well known CTCF binding site and insulator element within the *HoxA* locus [47••,51]. A similar result was obtained by comparison of ChIP-seq and 5C data in mESCs [48••], and for *Drosophila* Kc167 cells and embryonic nuclei [50•,52]. However, CTCF binding alone appears to be insufficient to specify the topological domain boundaries, since over 85% of the CTCF binding sites are found within topological domains [47••]. Further examination of domain boundaries revealed an enrichment of highly transcribed sequence features particularly housekeeping genes, tRNAs, and SINE elements [47••,52]. These findings suggest that transcriptional activity may also play a role in boundary formation. Whether CTCF binding and transcription actively contribute to formation of topological domains, and if so, how, will need to be addressed in future experiments.

Domains as co-regulated units or blocks are evolutionarily and developmentally conserved; however, the activity of genes and regulatory elements within these domains can be highly dynamic. Throughout differentiation entire TADs can become Lamin associated, suggesting a large-scale change in nuclear positioning. During B-cell differentiation domains containing several genes, including *Ebf1*, are in repressive regions in pre-pro-B cells near the nuclear

lamina but repositioned to active regions in pro-B cells [49]. Sexton *et al.* demonstrated that in *Drosophila* embryonic nuclei inactive domains are sequestered away while active domains undergo more frequent intra-chromosomal and inter-chromosomal interactions [50•]. In Kc167 cells, active domains were also found to have distinct physical locations within the nucleus, with repressive domains at the periphery and active ones more centrally located. Moreover, the domain boundaries in Kc167 cells are involved in long-range interactions, perhaps also functioning to cluster active and coordinately regulated genes together [52].

Within each topological domain, it is unclear what could contribute to the formation of more dynamic chromatin structures. It is possible that CTCF and cohesin play a role in this process as they have been found to mediate looping at the immunoglobulin, HoxA, β-globin, and interferon gamma loci [46,53]. In contrast, in B-cells, CTCF binding correlated with invariant intradomain interactions, whereas enhancer associated factors p300, E2A, and PU. 1 correlated with dynamic, developmentally regulated, interdomain interactions [49]. In addition, 5C analysis revealed that long-range interactions frequently span CTCF binding sites [40••]. CTCF could be functioning as an insulator or barrier in repressed regions but also in a positive manner in active regions looping enhancers and promoters together. While CTCF plays numerous roles in both large-scale and small-scale domain structures other factors are also at work. Despite being commonly linked to CTCF, Cohesin has recently been ascribed a CTCF-independent role in transcriptional regulation and chromatin looping. Binding of Cohesin, and its loading factor Nipbl (Nipped-B in *Drosophila*), to the LCR of the β-globin locus was shown to be critical for long-range enhancer interactions and for gene expression [54]. In corroboration of this, Nipped-B was previously identified in a *Drosophila* genetic screen for factors controlling enhancer–promoter communication [55]. ChIP-seq analysis in mouse liver samples demonstrated that Cohesin binding correlates with binding of TFs, master regulators, and H3K4me1 at enhancer loci. Cohesin localization also correlated well with liver-specific gene expression. Furthermore, analysis of Rad-21 haploinsufficient liver cells suggests that Cohesin acts to stabilize protein-DNA complexes important in looping [56]. In addition, Cohesin and Nipbl were found linked to Mediator in a screen for factors regulating ESC pluripotency and Oct4 expression. ChIP-seq binding analysis reveals Mediator and Cohesin binding, along with Nipbl, at promoters and enhancers of ESC specific genes such as *Oct4* and *Nanog*. Binding of these factors correlates with enhancer promoter communication at these sites and is dependent on Nipbl association. KD of Cohesin or Mediator led to a decrease in looping and a concomitant decrease in gene expression [57].

Future perspectives

The evidence presented here highlights a complex process to bring about association of enhancers with promoters for the correct spatio-temporal expression of genes (Figure 3). A recent advance in our understanding of the chromatin organization is that the genome is partitioned into inherently stable ‘topological domains’ that serve to demarcate functional regulatory blocks. Multiple lines of evidence suggest that topological domains may play a critical role in dictating the communications between enhancers and promoters. Nevertheless, topological domains should be viewed as only one layer of a more complex

picture. It is very likely that there are further subdivisions within each topological domain. Further, topological domains may organize into higher order structures that could correspond to large-scale features of the nucleus. Our knowledge of 3D organization needs to be integrated with transcription factor binding and transcriptome data to more specifically understand the gene regulatory networks in mammalian cells. While we have made great inroads into the role of genome architecture in gene regulation, it is still unclear whether all enhancer–promoter interactions are functional. Future experiments should be focused on demonstrating the functional relationships between long-range chromatin interactions and gene regulation. With knowledge of location and functionality of interactions between regulatory elements in hand we will be poised to decipher the gene expression programs encoded in the genome.

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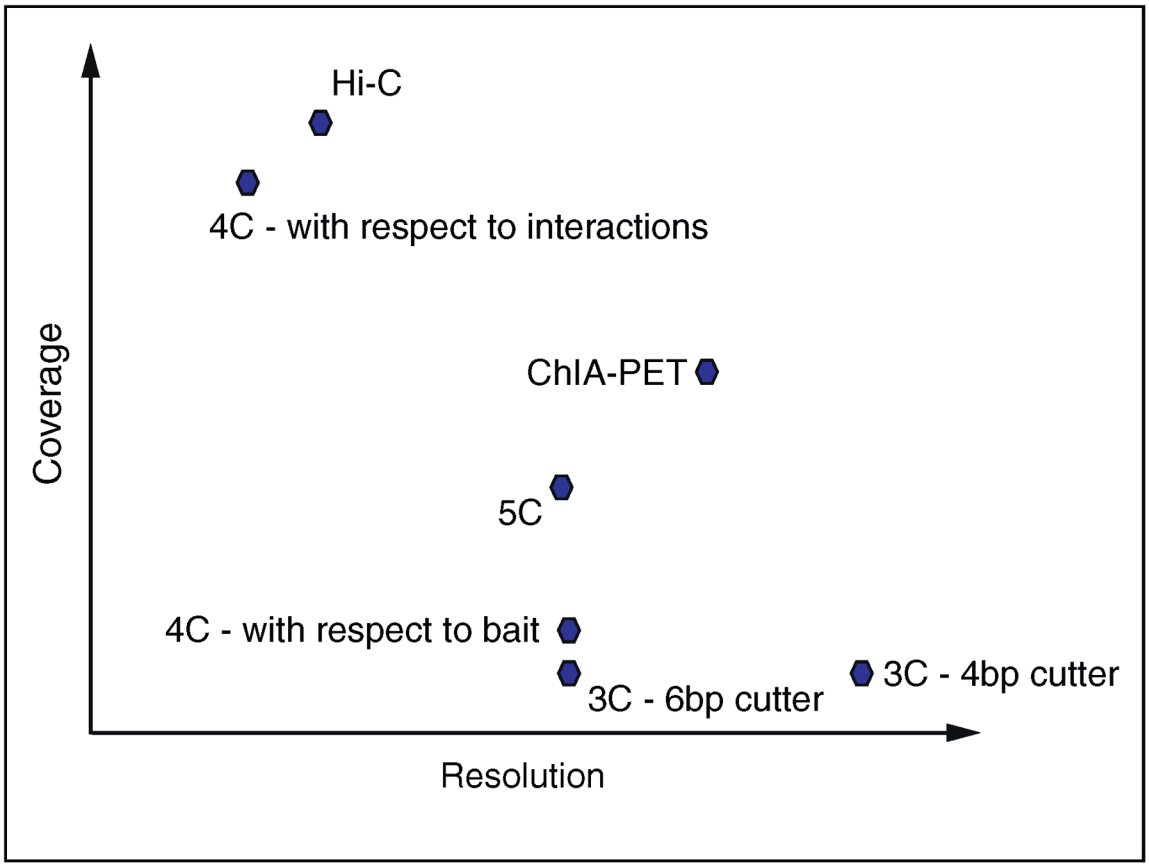

Figure 1.
Comparison of coverage versus resolution for chromatin conformation capture techniques.

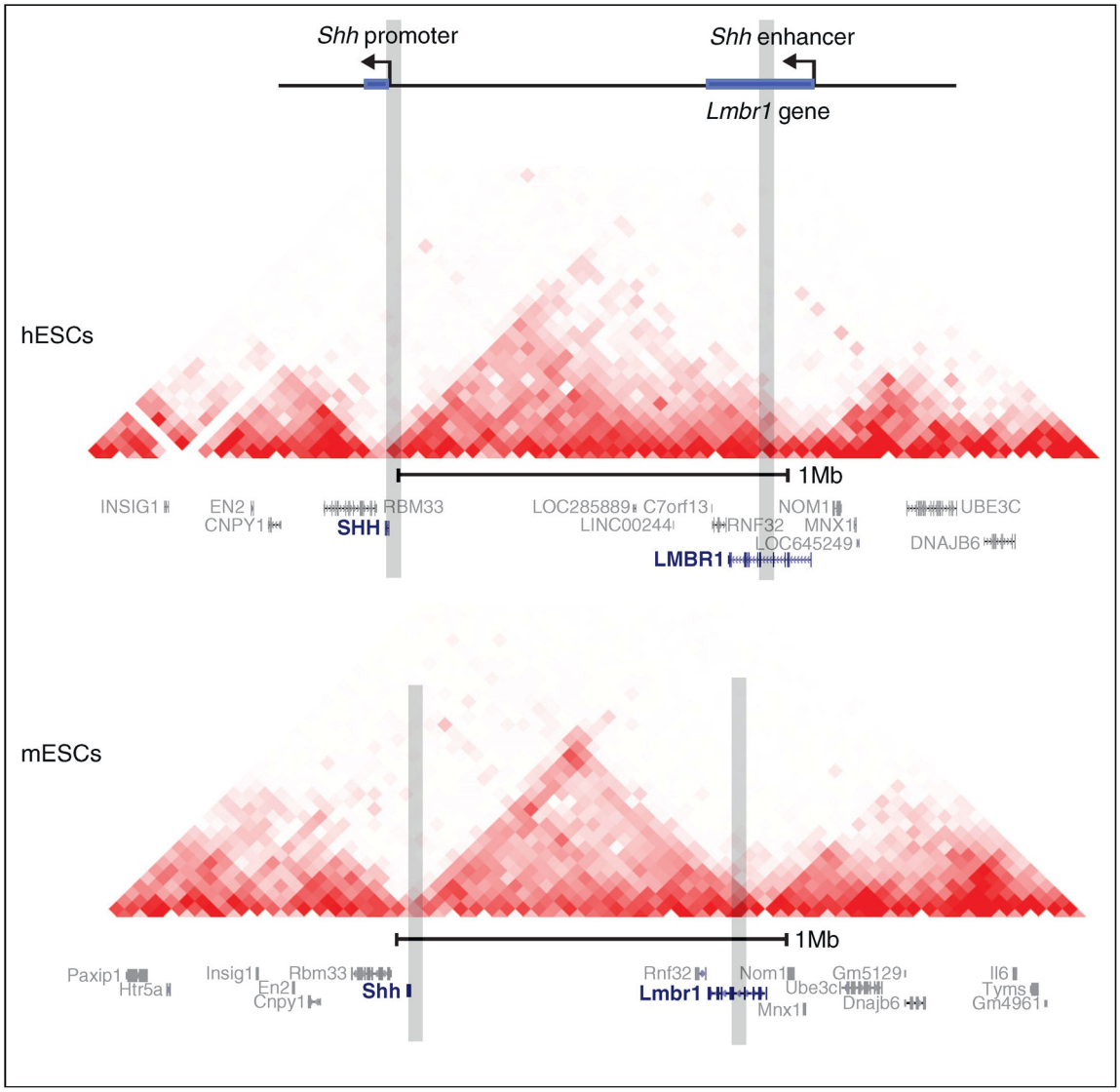

Figure 2.

Hi-C analysis of mouse and human ESCs. The *Shh* enhancer and gene, marked by grey shaded boxes, lie ~1 Mb apart but contained within a conserved topological domain to allow for correct regulation of gene expression.

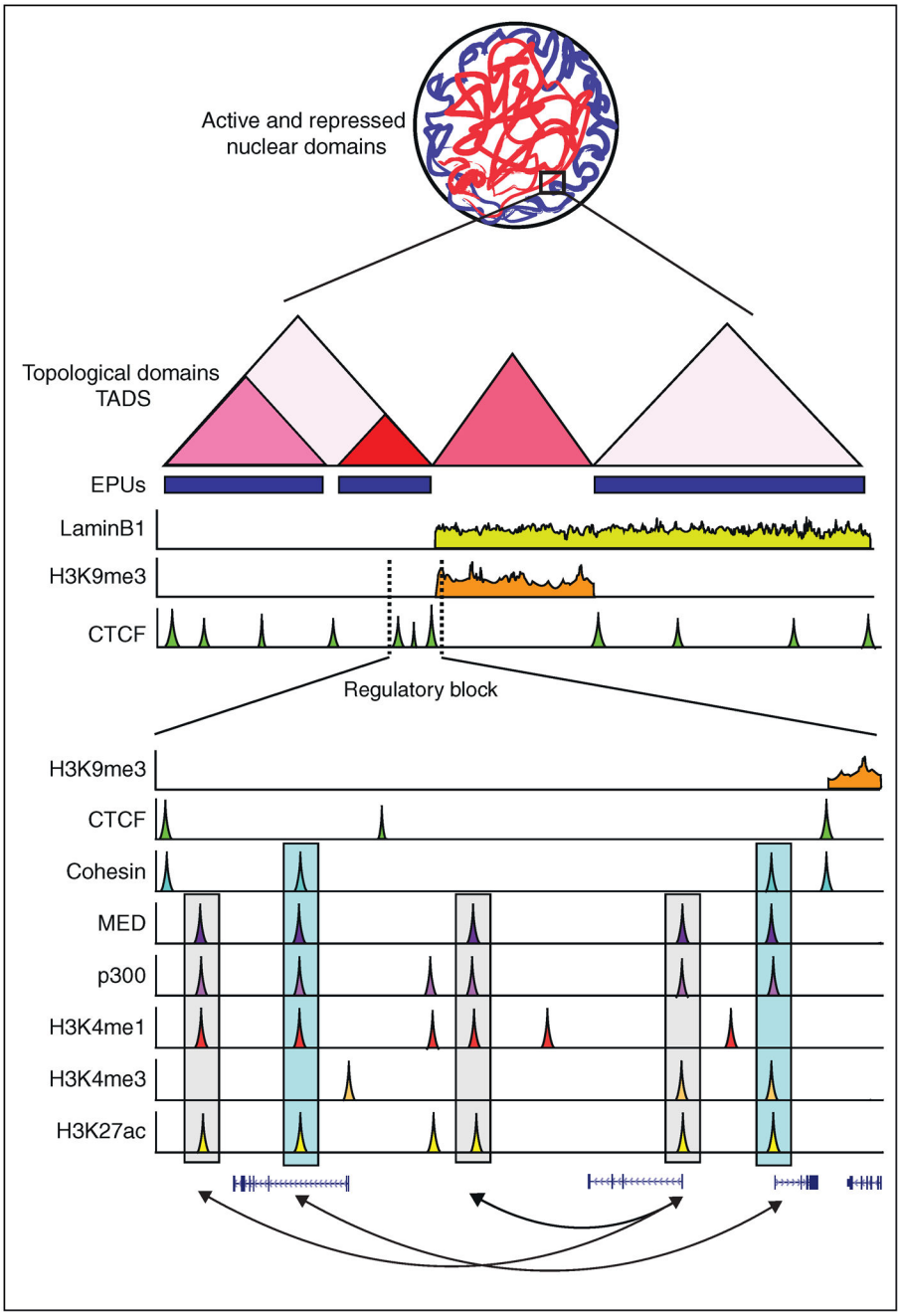

Figure 3.

Schematic of a layered genomic organization. Top: Active and repressed regions are sequestered into separate compartments with active domains frequently looped out into the interior of the nucleus. Middle: At a higher resolution, the genome is organized into self-associating regulatory blocks or topological domains. These domains can be associated with LaminB1 and function to contain the spread of repressive regions of histone modifications during differentiation and development. The boundaries are frequently found associated with CTCF and transcribed genes. Bottom: Within a specific regulatory block active promoters

and enhancers are held in proximity to increase the likelihood of association. Cohesin and Mediator have been implicated in enhancer–promoter looping in this context.