Opinion TRENDS in Genetics Vol.22 No.4 April 2006

Chromatin looping and the probability of transcription

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Recent studies of several multigene clusters have shown that gene activation by a remote enhancer is associated with chromatin loop formation. It is not fully understood how a chromatin loop forms in a nucleus or how it is involved in gene regulation. In this article, we propose that the major feature that determines loop formation is the flexibility of chromatin, and that this flexibility is modulated by histone acetylation (and other modifications). Thus, histone modifications will modulate distribution of the preferential looping site in chromatin, which, in turn, determines the probability of interaction between a remote enhancer and the cognate genes. This model can explain gene expression changes in the *Hoxd* gene cluster and the β–globin locus.

Introduction

Numerous studies suggest that chromatin accessibility (chromatin opening) to trans-activating factors is the major mechanism by which chromatin regulates gene activity [1,2]. The 'histone code' or 'histone surface' hypotheses (see Glossary) implies that chromatin not only regulates accessibility but also regulates binding specificity through altering various covalent modifications of the histone tails [3–7].

Although studies in several multigene clusters have shown that gene activation by a remote enhancer is associated with chromatin loop formation [8,9], the nature of its formation and involvement in gene regulation is not fully understood. We propose that the major feature that determines loop formation is the chromatin flexibility, which is modulated by histone acetylation (and other modifications). Therefore, histone modifications lead to the determination of the probability of interaction between a remote enhancer and its cognate genes. These features constitute the main elements of the facilitated chromatin-looping hypothesis.

It has long been disputed how an enhancer activates its cognate genes across distances up to ~800 Kb. To date, four different, but not mutually exclusive, hypotheses have been put forward (Figure 1). (i) Chromatin looping between the enhancer and promoters is a simple and attractive model [10–12]. (ii) A tracking or scanning model hypothesized that the transcription-activating complex recruited by an enhancer linearly tracks along chromatin until it encounters a competent promoter [13]. In this model, the scanning process will not alter the proximity between the enhancer and the promoter. (iii) The facilitated tracking model incorporates aspects of both the looping and tracking models [14]. An enhancer and its activation complex migrates along the chromatin fiber until it encounters the cognate promoter. The intervening chromatin between the enhancer and the promoter 'reels out' through the enhancer complex and forms a loop, which is progressively enlarged during tracking. (iv) The linking model proposes that the binding of facilitator proteins between an enhancer and its cognate gene defines the activated domain and mediates transcription-enhancing activity [15].

Formulation of the facilitated chromatin-looping hypothesis

Close proximity of a distal enhancer to its active cognate gene

Recent studies have shown that a distantly encoded enhancer can be located close to the active gene, suggesting that a chromatin loop forms between them. Using chromosome conformation capture (3C) and tagging and recovery of associated protein (RNA TRAP) assays [8,9,16], it was shown that in erythroid cells the hypersensitive sites of the locus control region (LCR), which are located 40–60 kb from the active genes, come close to the active genes. By contrast, the globin cluster adopts a seemingly linear conformation in brain. Formation and/or stabilization of the interaction require trans-acting factors, such as erythroid Kruppel-like factor (EKLF), GATA-1 and Fog-1 (a zinc finger protein) [17,18]. Using the same approaches, it was shown that a chromatin loop is formed between the T_H2 LCR and its cognate genes encoding interleukin (IL)-4, IL-5 and IL-13 in T-helper type 2 cells [19] and between three Vκ enhancers, which are active gene promoters, and a 3′ boundary element in B cells [20]. In addition to enhancer–promoter loops, parent-specific loop formation was observed between the differentially methylated regions in imprinted insulin-like growth factor 2 and H19, a maternally expressed untranslated mRNA (*IGF2/H19*) [21].

Taken together, these studies show that chromatin looping brings cis-regulatory elements close together. But the mechanism that modulates chromatin looping remains poorly understood.

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Available online 21 February 2006

 0168-9525/$ - see front matter doi:10.1016/j.tig.2006.02.004

Glossary

Histone surface hypotheses: histone tails can be modified by acetylation, methylation, phosphorylation and ubquitination, which, in turn, affect the accessibility of the chromatin to various proteins.

The facilitated chromatin-looping hypothesis: implies that gene activation is regulated not only by chromatin accessibility and binding specificity modulated by histone modifications, but also by the probability of interaction between an enhancer and its cognate promoter.

The flexibility of chromatin fiber modulates loop formation

Assuming that both ends of a large section of chromatin are anchored at a nuclear substructure, what is the probability of contact between two points in the section? A biophysical consideration of this question is given in the supplementary material online. The probability of loop formation between two points in a linear fiber is determined by the size of the chromatin section, the distance between the two points (i.e. enhancer and gene) and the flexibility of the chromatin fiber. In a genome, the distances between an enhancer and its cognate genes are fixed. The size of the chromatin section anchored at nuclear substructure (e.g. the nuclear matrix) is probably also fixed in a given cell type. Therefore, the major determinant that governs the probability of interaction between an enhancer and the cognate gene is flexibility. We conclude that: (i) when chromatin flexibility is constant, the optimal interaction distance is fixed, meaning that the activation probability of a gene by a distal enhancer is determined by the distance between them; (ii) when chromatin flexibility changes, the gene that can be maximally activated by an enhancer will be changed accordingly.

Ringrose *at al.* showed the importance of flexibility in determining the interaction frequency between two points in linear DNA in the recombination between FLP and its recombination target (FRT) [22]. They found that the percentage of recombination between two FRT sites, catalyzed by FLP DNA recombinase, was dependent on the distance between the two sites but independent of the FLP enzyme concentration. The degree of DNA flexibility calculated from their data was consistent with that of naked DNA, suggesting that the DNA flexibility solely determines the contact frequency between two FRT sites. A similar conclusion was reached using an *in vivo* FTP recombination assay, which suggested that the flexibility of chromatin is approximately twofold greater than that of naked DNA [22].

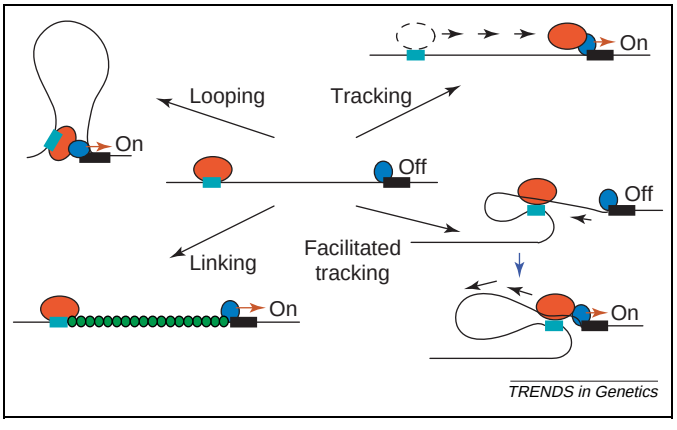

Figure 1. How does a remote enhancer activate its cognate gene over a large distance? Four different models, looping, tracking (or scanning), facilitated tracking and linking, have been proposed to explain this phenomenon. Each model implies that the activation complex recruited by an enhancer meets the promoter, except the linking model, which proposes that the 'linking' proteins mediate the communication between an enhancer and its cognate gene. Sky blue rectangles represent an enhancer, the activation complex recruited by the enhancer is shown in red. The genes are represented in black and the complex formed on the gene promoter is represented by the blue circles. The linking proteins (e.g. Chip protein in *Drosophila*) are shown in green.

Histone acetylation alters chromatin flexibility

What can alter the chromatin flexibility in the nucleus? We hypothesize that acetylation of histones (and other histone modifications) changes chromatin flexibility. Several biophysical studies have provided evidence supporting this idea. Evidence that the core histone termini are required for all aspects of nucleosomal array condensation comes from studies showing that arrays assembled from trypsinized histone octamers lacking their N-termini domains can neither fold nor oligomerize in the physiological salt range [23,24]. Histone hyperacetylation leads to a 1.1–1.8-fold increase in equilibrium accessibility of nucleosomal DNA target sites [25]. A low acetylation threshold level could destabilize higher order folding to the same extent as the proteolytic removal of all N-termini [26]. In the physiological salt range and in the absence of histone H1, the acetylated oligonucleosome complexes remain in an extended conformation, in contrast to their non-acetylated counterparts [27]. Reconstituted acetylated chromatin had an increased sensitivity towards DNase I and, surprisingly, an increased degree of conformational flexibility following a change in temperature, indicating profound alterations of DNA-histone interactions [28].

Collectively, these results suggest that histone acetylation tends to weaken higher order chromatin structure and increase fiber flexibility.

Experimental evidence

Hoxd gene cluster

If flexibility is constant, the probability of an interaction between any two points is defined. Thus, the probability of a gene being activated is determined by the distance between the gene and its cognate distal enhancer. This conclusion is supported by studies of gene inactivation (gene deletions or promoter mutations) in the *Hoxd* cluster. The *Hox* genes are needed for the development of all bilaterally symmetric animals [29]. In higher vertebrates, these genes are grouped into four clusters, *Hox*A, *Hox*B, *Hox*C and *Hox*D. Vertebrate *Hox* genes are arranged and expressed in the same order as the parts of the body they help to produce. In addition to this collinear order of expression, *Hox* genes have 'quantitative collinearity'; the genes at one end of the cluster (5′) are expressed at the greatest levels, whereas those located closer to the 3′ end are expressed at progressively lower levels. The position of a gene in a cluster correlates not only to its place and time of expression but also to its expression level. The molecular mechanism of

'quantitative collinearity' is not yet fully understood, however this pattern of gene expression can be interpreted by the facilitated chromatin-looping mechanism.

A subset of mouse *Hoxd* genes (*Hoxd10–Hoxd13*) are required for limb development. A digit enhancer in the global control region (GCR) is ~100-kb 5′ to *Hoxd13* and regulates expression of these genes [30]. Similar to other *Hox* genes, these genes are expressed in a quantitatively collinear manner. *Hoxd13* is closest to the 5′-end of the cluster and is expressed most anteriorly and at the greatest level; *Hoxd12*, *Hoxd11* and *Hoxd10* are expressed at progressively lower levels and in more posteriorly restricted domains (Figure 2). If the activation of these genes depends on interaction between the digit enhancer and the genes, and this interaction is mediated by chromatin looping, the pattern of transcription of the genes will be determined by distribution of the preferential looping sites (Figures 2 and S1 in the supplementary material online), resulting in a linear decrease in transcription from *Hoxd13* to *Hoxd10* as shown in Figure 2a.

A large-scale study of mutations, deletions and duplications in the mouse *Hoxd* cluster confirmed that this occurs [31]. Inactivation of *Hoxd13* by the promoter mutation led to an overall reduction in the size of digits, partial fusion between digits III and IV and, in most cases, an extra digit. By contrast, the deletion of *Hoxd13*, which is closest to the 5′ end, generated a mild alteration of digit morphology in homozygous animals. In the *Hoxd13*$^{-/-}$ mutant, a strong gain of expression of *Hoxd12* was found, resembling the expression pattern of *Hoxd13*. The facilitated chromatin-looping model interprets the phenotypes as follows: both promoter inactivation and gene deletion inactivate the corresponding gene, the inactivation mutation does not change the genomic distance between the enhancer and genes, whereas deletion mutation does. The *Hoxd13* inactivation mutation will abolish expression of the gene, but will not affect the expression pattern of other genes because the distribution of the preferential chromatin-looping sites remains the same (Figure 2b,ii). Thus, the loss of *Hoxd13* product will create a much more profound phenotype. However, the deletion mutation will bring *Hoxd12* to the *Hoxd13* position (Figure 2b,iii). The facilitated chromatin-looping mechanism predicts that *Hoxd12* and *Hoxd13* will be expressed at similar levels, and the loss of *Hoxd13* product will be compensated for by the product of *Hoxd12*. As the result, the phenotype that results from the *Hoxd13*$^{-/-}$ mutation will be mild. Similar changes in gene expression patterns were observed when both Hox genes were either deleted or inactivated. When both *Hoxd13* and *Hoxd12* were deleted, *Hoxd11* and *Hoxd10* moved to the original positions of *Hoxd13* and *Hoxd12*, resulting in *Hoxd13*- and *Hoxd12*-like expression patterns for *Hoxd11* and *Hoxd10* (Figure 2b,v). When *Hoxd13* and *Hoxd12* were both inactivated, the expression pattern of *Hoxd11* remained unchanged because the relative distance between the digit enhancer and the gene was retained (Figure 2b,iv). However, the double deletion resulted in severe morphological changes, indicating that HOXD11 cannot be a functional substitute for either HOXD13 or HOXD12. Therefore, all these phenotypes are consistent with the prediction of the facilitated chromatin-loop mechanism.

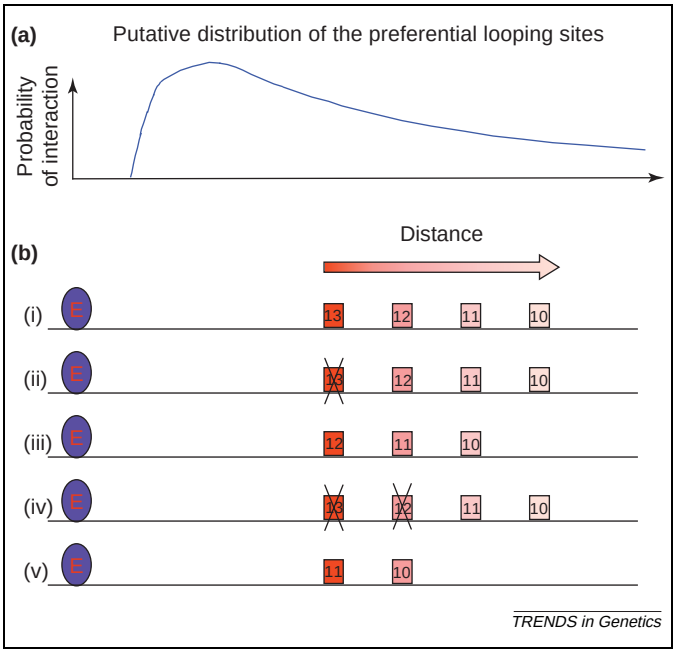

Figure 2. The facilitated chromatin-looping model can be used to interpret the different effects of gene inactivation and gene deletion on *Hoxd* gene expression. **(a)** A putative curve of the preferential looping sites (see Figure S1 in the supplementary material online). **(b)(i)** In the wild-type locus, *Hoxd13* supposedly situates near the peak of the putative preferential looping curve, ensuring that the gene is expressed at the greatest level. The expression levels of the downstream genes (*Hoxd12, Hoxd11* and *Hoxd10*) progressively decline corresponding to their locations. **(ii)** Inactivation of *Hoxd13* does not alter the geometric relationship between the digit enhancer (E) and the genes. Therefore, the expression level of *Hoxd12* remains unchanged. **(iii)** However, when *Hoxd13* is deleted, *Hoxd12* moves to the original position of *Hoxd13*, resulting in a *Hoxd13*-like expression for *Hoxd12*. The same argument can be applied to the *Hoxd13* and *Hoxd12* double inactivation and deletion mutation (**iv** and **v**).

RXII is an evolutionarily conserved region with no defined function, which is located 5′ to *Hoxd13*. A 1.2-kb deletion of RXII combined with the *Hoxd13* deletion led to a gain of expression for all three downstream genes [31]. On the basis of this phenotype, the facilitated chromatin-looping hypothesis predicts that the function of the conserved RXII region could be related to histone modification enzymes. Deletion of RXII would result in a redistribution of the preferential looping curve, so that the peak of the preferential looping position shifts to the three downstream genes.

Based on the facilitated chromatin-looping hypothesis, the linear decrease pattern in transcription from *Hoxd13* to *Hoxd10* results from the flat-level distribution of histone acetylation within the region encompassing the digit enhancer and the *Hoxd* genes. Although, a comprehensive histone acetylation profile in the murine *Hoxd* region has not been reported, available data demonstrated that histone H3 at the *Hoxb1* and *Hoxb9* genes were acetylated at similar levels in ES cells and treatment with retinoic acid induced changes in the acetylation levels in both genes, but to a similar extent [32]. Our hypothesis also anticipates that a continuous domain of histone acetylation should be present in the *Hoxd* cluster. The presence of a broad histone H3 lysine 4-methylated domain overlaying multiple active genes in the human

and murine *Hoxa* clusters [33] is consistent with our proposed model.

The β-globin locus

The human β-globin locus is ~100 kb on chromosome 11, with a distal enhancer or LCR and five functional genes; ε, $^{G}\gamma$, $^{A}\gamma$, δ and β, organized in the order of their developmentally timed expression (Figure 3a) [34]. The ε-globin gene is the first to be expressed during ontogeny; its expression is restricted to the blood island of the yolk sac. At ~6–8 weeks of gestation, expression of ε-globin is silenced and expression of the γ-globin genes is activated in fetal liver. At the time of birth, approximately, δ- and β-globin gene expression increases dramatically, accompanied by a shift of erythropoiesis to the bone marrow. At the same time, the γ-globin gene expression declines progressively and is finally silenced at ~1 year old (Figure 3b), a process known as hemoglobin switching. We propose that hemoglobin switching results from changes in preferential looping sites between the LCR and the globin genes due to alterations of the distribution of histone acetylation during the course of development.

The distribution of histone acetylation in the human and mouse β-globin loci has been studied by several groups [35,36]. The LCR was found to be heavily acetylated at all stages of development, whereas elevated levels of acetylation in the regions containing the globin genes were associated with gene activation. Similar results were obtained from human primary erythroblasts (G. Barkess *et al.*, unpublished). The LCR is heavily acetylated in primary fetal and adult erythroblasts (Figure 3c,e), suggesting that differentiation during erythropoiesis does not affect the status of histone acetylation. In fetal erythroblasts, the ε gene, which is silenced in fetal liver, is either not acetylated or lightly acetylated; the γ genes, which are highly transcribed, are heavily acetylated; and the δ and β genes, which are expressed at low levels, are slightly acetylated (Figure 3c). In adult erythroblasts, the heavily acetylated region encompasses the δ and β genes, which are the predominantly transcribed globin genes in adult, whereas the regions encompassing the ε and γ genes are lightly acetylated (Figure 3e). According to the relationship between histone acetylation and chromatin flexibility discussed in the supplementary material online, the preferential looping profiles based on these acetylation patterns could be roughly depicted as in Figure 3d,f. Histones in the ε gene region are not acetylated in fetal liver; therefore, the chromatin flexibility is expected to be low. This less flexible region constrains chromatin to form a small loop, but favors contact between the LCR and the nearest γ gene (Figure 3d). In adult erythroblasts, the ~30-kb region between the ε and γ genes is either not acetylated or lightly acetylated, which forces chromatin to form a large chromatin loop and results in predominant activation of the δ and β genes. Hence, hemoglobin switching is consistent with the facilitated chromatin-looping model.

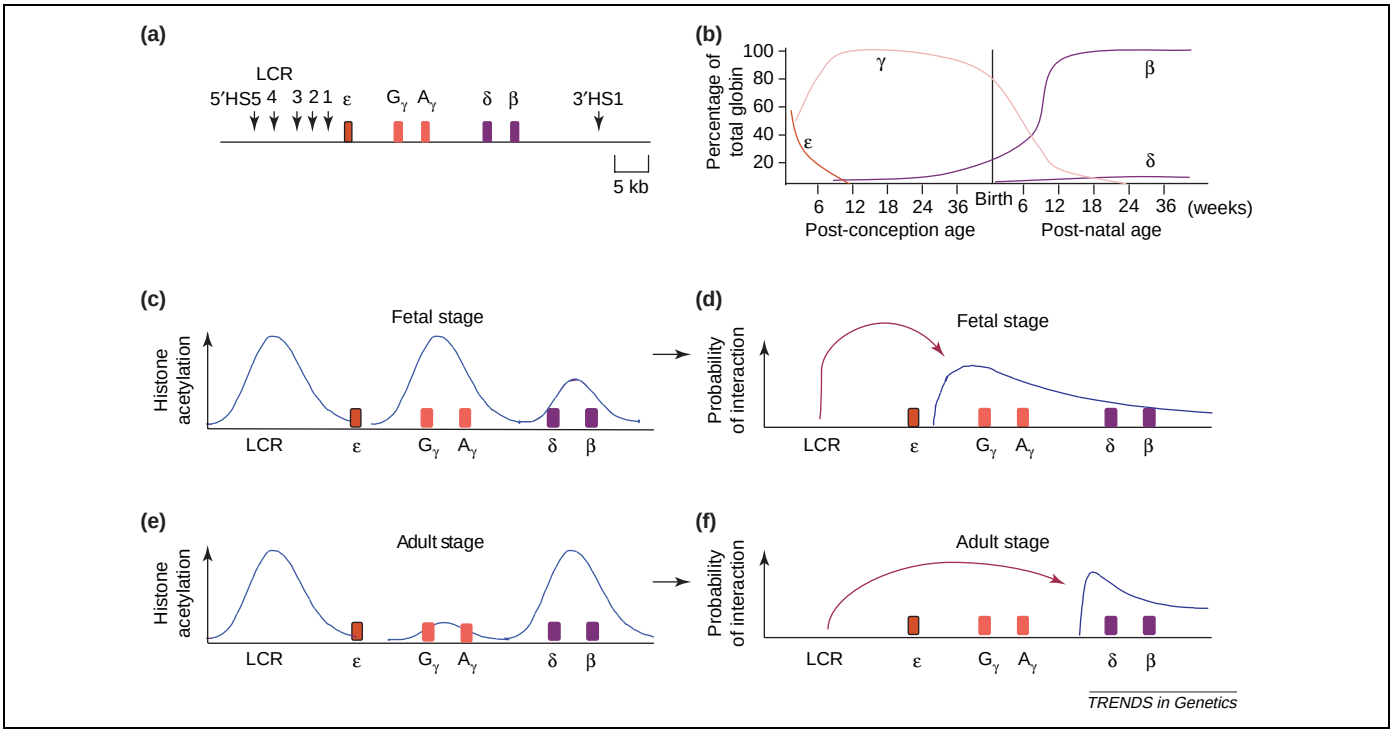

Figure 3. The relationship between histone acetylation, chromatin flexibility and hemoglobin switching. **(a)** The human β-globin locus. The LCR consists of five DNase I hypersensitive sites (HSs 1–5) and functions as a powerful erythroid-specific enhancer. **(b)** The relative transcriptional levels of the β-like globin genes during the course of developmental. **(c,e)** The distribution of histone acetylation in the β-globin locus in fetal and adult developmental stages. The LCR region is always heavily acetylated in human primary fetal liver and adult erythroblasts. In the regions encompassing the globin genes, acetylation profiles are developmental-stage specific. In fetal liver the γ–globin gene region is heavily acetylated, but not the ε –globin gene region. In adult erythroid cells, the δ– and β–globin regions are heavily acetylated, but not the ε– and γ–globin gene regions. **(d,f)** Based on these distributions and the relationship between acetylation and flexibility, the probability of looping position between the LCR and the regions containing the globin genes can be depicted by the blue curves as shown (see also Figure 2b in the supplementary material online). Although this is not a mathematical conversion because of the lack of qualitative parameters, the conceptual translation holds true. The preferential looping site is situated close to the γ-globin gene region at the fetal stage, and shifts down to the β-globin gene region in adults.

The facilitated chromatin-looping mechanism anticipated that a change in either the relative distance between an enhancer LCR and its cognate genes or of gene order will influence expression patterns. Effects of distance and gene order in the β–globin locus have been studied [37–40]. For example, when a marked (or mutated) β-globin gene replaced the ε gene in the context of the entire locus, the distance between the LCR and the marked β gene was ~10 kb and it was still ~50 kb for the wild-type β gene. The expression from the marked gene accounted for 88–99%, whereas only 1–12% came from the wild type β gene. Both the marked and wild-type β genes have the potential to be activated and acetylated in adult erythropoiesis. Based on the facilitated loop model, the flexibility of the region between the marked β gene and the LCR will be increased owing to the heavy acetylation of the marked β gene. Thus, the chromatin tends to form a small loop, but favors the interaction of the LCR with the nearby marked β gene, rather than with the wild-type β gene, which is 50-kb downstream. This mechanism can explain the competitive advantage of the proximal gene. The same argument explains the dominant expression of the β gene and suppression of all other genes when the 48-kb globin gene region spanning the ε and β genes is inverted in βYAC transgenic mice [41].

Concluding remarks

3C and RNA-TRAP assays determine the final status of chromatin looping but do not monitor the looping process. Before a loop forms, many mechanisms can alter chromatin flexibility. For example, an enhancer-recruited activation complex could contain some kind of histone modification activities. When it 'tracks' on chromatin, as proposed in the tracking or facilitated tracking models, the complex can modify histones, resulting in an alteration of the chromatin flexibility. Linking proteins, proposed in the linking model, or the transcription complex, which is responsible for production of intergenic RNA, could carry or recruit histone modification enzymes. Thus, many components associated with the tracking, facilitated tracking, linking and intergenic transcription models can be integrated into the facilitated chromatin-looping mechanism.

Eukaryotic genes are regulated by several mechanisms. The modulation of chromatin accessibility and the specificity of trans-factor binding regulates the possibility of gene activation. The idea that histone covalent modifications modulate the probability of interaction between two points in a chromatin adds a novel layer of regulation on top of chromatin accessibility and binding specificity. We do not know the quantitative relationship between chromatin flexibility and the level of histone acetylation and other modifications. In addition, the flexibility probably fluctuates within a section of chromatin. A precise description of the chromatin behavior in a nucleus is impossible based on current experimental data and theoretical analysis. However, the facilitated chromatin-looping mechanism provides a prototype model that explains the probability of gene activation through histone modifications, and thus chromatin flexibility. It might also provide a means to translate genomic linearity into transcription gradients in eukaryotes.

Acknowledgements

We thank Sean Kincaid and Anh Ngo for their critical reading of this article. This work is supported by NIH grants DK61805 and HL073439 to QL.

Supplementary data

Supplementary data associated with this article can be found at doi:10.1016/j.tig.2006.02.004

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