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Boundaries. Boundaries...Boundaries???

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One way to modulate transcription is by partitioning the chromatin fiber within the nucleus into the active or inactive domains through the establishment of higher-order chromatin structure. Such subdivision of chromatin implies the existence of insulators and boundaries that delimit differentially regulated chromosomal loci. Recently published data on transcriptional interference from the repeated component of the genome fits the classic definition of insulator/boundary activity. This review discusses the phenomena of transcriptional interference and raises the question about functionality of genomic ''junk'' along with the need to stimulate a dialogue on how we would define the insulators and boundaries in the light of contemporary data.Rule 19 (a) (Boundaries)''Before the toss, the umpires shall agree the boundary of the field of play with both captains. The boundary shall, if possible, be marked along its whole length''Rules of Cricket

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'Boundaries' are by their very nature, complex constructs of man. The use of the term 'border' can mean an international line or a region encompassing both sides of a political boundary. The Treaty of Guadalupe Hidalgo delineated specific boundaries between what was to be San Diego and Tijuana. Yet today, despite the presence of the barb-wired demarcation line, the exact boundary of the border remains a blur. The reason is simple. In a sense, the 'boundary' is a subjective construct, meaning many things to many different people. The same is true for genomic boundaries.

Imagine that genes within the genomes not only have defined 'addresses' (specific positioning within the specified chromosome) but also are assigned to 'neighborhoods'. Chromatin within the cells is organized based on its functionality [1]. Gene neighborhoods are defined as a convex of physical (functional) areas containing similar epigenetic landmarks and roughly homogeneous principles for their regulation [2–4]. In a very simplified way these neighborhoods can be graded from 'highly active' to 'transcriptionally poised' and 'silenced', and such grading correlates with particular nucleosome arrangements, histone variants, histone modifications, and interactions of nonhistone regulators [5•,6,7•,8–11]. Active **euchromatin** and genetically inert **heterochromatin** are differentially positioned within the eukaryotic nucleus, and this positioning could reflect distinct environments within the nucleus designated for chromatin activation and repression, respectively [12–18]. Residence in a gene-dense neighborhoods as well as transcriptional activity seem to be important factors to determine gene positioning [17]. The nuclear interior homes most of transcriptionally competent chromatin [19]. The nuclear periphery is strongly connected to gene silencing and chromatin 'close' conformation [20].

Several models were proposed to explain interphase functional chromosomal domains organization and gene expression based on these novel facts [21–23]. Interestingly enough, chromatin loops may exhibit considerable motion within the nuclear volume. Loop movements probably allow loci repositioning in order to approach functional transcription sites or repressive nuclear environments. It has been suggested that active chromatin loops may merge into nuclear regions of high gene expression or euchromatic 'neighborhoods' maximizing cell transcriptional capabilities [24]. Extensive intrachromosomal and interchromosomal associations (via chromatin looping) throughout the genome bringing into proximity distant genes or gene regulators have also been reported [19,25,26]. One of the sticking examples published recently describes the genome positional flexibility of olfactory receptor genes dispersed in the several chromosomes in each neuron [27]. Another impressive example is the interaction between ER-regulated genes allocated on chromosome 2 and 21 in human cell line [28]. Upon treating cells with estradiol, ER-regulated genes from the different chromosomes are experiencing long-range movement and repositioning into the specialized nuclear compartments enriched with ER–co-regulators. This nuclear rearrangement is required to ensure an appropriate level of transcription activity and support the hypothesis of the specialized factor-enriched compartments within the cell nucleus.

Taking into consideration such complex and dynamic three-dimensional organization of the chromatin within the nucleus, it has become reasonable to raise the ques-

tion of how the chromosomal domains integrity can be established during development and cellular differentiation and how is it mediated during cell divisions? The concept of genomic boundaries was introduced in order to delimit discrete and topologically independent high-order domains [29,30]. It has been postulated from early 1970 that similar to the geographic borders, genomic boundaries might play a role in the organization of the chromatin fiber into the functional domains, such that genes present in one domain are not affected by regulatory sequences present in a different one. This dazzling concept of such 'molecular bookmarks' is subjected for few decades of investigations, debates, and surely enough, is not without controversy. While numerous boundary sequences have been identified in organisms, ranging from yeast to humans [29–32], the question of whether they are merely structural components or whether they play a functional role in the expression of particular genes is still unanswered.

Molecular models of the boundaries action

What do we imply by claiming boundaries? The term 'boundary' describes a phenotype read-out of the experimental assays for its identification and characterization rather than a single kind of element with a fixed mechanism of action.

There are two defining properties of boundary elements. The first property is their 'insulation' ability to block the action of a distal enhancer on a promoter [33,34]. Enhancer blocking occurs only if the insulator is situated between the enhancer and the promoter, not if it is placed elsewhere. The reader is directed to other excellent reviews covering enchancer-blocking insulators and molecular players mediating their functions [35•,36]. The second property, in which boundaries could be defined, is by acting as 'barriers' against self-propagating heterochromatic position effects [32], thus preventing the advancement and spreading of nearby condensed chromatin that might otherwise silence gene expression within euchromatic domain. Some boundaries are able to act both as enhancer-blocking insulators and barriers. No common sequence features are apparent among characterized chromatin barriers. In accordance with barrier DNA sequences, chromatin barrier proteins are also heterogeneous, though increasing evidence suggests that the recruitment of histone acetylase activity correlates with barrier activity in multiple organisms [37–39].

There are several ways in which individual chromosomal 'neighborhoods' could maintain independence from their surroundings through the establishment of the boundaries. First, specific DNA sequences and associated proteins might have the role of establishing fixed boundaries. Second, boundaries might not necessarily have a sequence conservation, might be variable in position, and being established through a balance between colliding chromatin modifying activities responsible for chromatin condensation on one hand and chromatin decondensation on the other. The third concept, is the phenomena of transcriptional interference from small, nonprotein coding transcriptional units (*tRNA* genes in yeast, SINE/Alu repeats in mouse) that might delineate the chromosomal boundaries. In this example a barrier in the surprising way works as a combination of two aforementioned strategies: first, it is a sequence of significant conservation in variety of the genomes with an ability to recruit DNA specific factors (Pol II and Pol III machineries); second, it might interfere with the propagation of heterochromatin by creating an obstacle, 'a nucleosomal gap', over these small transcriptional units or by serving as an entry site for chromatin remodeling activities [16••,40]. This type of barrier activity, at least in mammalian genome, can be turned on/off in a developmental or signal-specific way, thus allowing plasticity in the chromosomal domains organization in cell-type or tissue-specific fashion [16••].

Barrier activity established by transcriptional interference from tRNA gene in yeast

In *Saccharomyces cerevisiae* yeast, the telomeres and the cryptic mating-type loci *HML* and *HMR* represent silenced chromatin domains. Studies of all three loci have revealed different barrier elements that block the spread of silencing these loci. The silenced *HMR* domain is flanked by long terminal repeats of *TY* elements, and the right flank of this silenced domain also contains a *tRNA* gene. A *tRNA* gene (tDNA) neighboring HMR is a principal component of the right-hand boundary of the silent chromatin domain [40]. The *tRNA* gene is distinguished by its ability to block silent chromatin from encroaching on the adjoining active chromosomal domain [41]. The transcriptional activity from tDNA creates a discontinuity in arrayed nucleosomes that acts as **a chain terminator** to the propagation of chromatin-bound Sir proteins [42]. Interestingly, not all *tRNA* genes in *S. cerevisiae* are equal in their ability to mediate barrier function. Only those that can mediate stable recruitment of TF IIIB complexes to their upstream Pol III driven promoters manifest the barrier activity, suggesting that occupancy of the promoters by Pol III machinery is important for the functional outcome [42]. In *S. cerevisiae*, additional proteins of the RNA Pol III transcriptional machinery are required and will be discussed later [40].

Genetic and biochemical studies using *S. pombe* as a model system have not only provided great insight into the mechanisms of heterochromatin assembly (for review see [14••]), but also verified the usage of transcriptional interference for the barrier function. The tDNAs within the pericentric repeat elements of *S. pombe* act similarly, serving as barriers to constrain pericentric heterochromatin [43••,44]. In these studies, authors demonstrate that $tRNA^{Ala}$ is actively transcribed and that disruption or deletion of the coding region results in the spread of

pericentromeric heterochromatin beyond its normal boundary. Moreover, inhibition of heterochromatin spreading is not dependent on centromeric location. TFIIIC, an RNA polymerase III (RNA Pol III) transcription factor, can form barriers independently of other RNA Pol III factors in *S. pombe*.

Analysis of histone modifications within insulated domains suggests that *tRNA* sequences might function by promoting or allowing the activity of histone-modifying enzymes that contribute to the maintenance of open chromatin. The enzymatic activity of these proteins might prevent the spreading of heterochromatin proteins from one compartment into the other. Support for idea that histone modifications can undergo changes at the $tRNA^{Ala}$ barrier comes from the demonstration that human TFIIIC relieves nucleosome-mediated repression and possesses histone acetyltransferase activity *in vitro* [45,46].

Taken together, these results are suggesting a common function of *tRNA* genes as genomic landmarks in single-celled eukaryotes. It is a common knowledge that many of the factors involved in heterochromatin formation in *S. pombe* are conserved in *Drosophila* and mammals. *tRNA* genes are also extremely conserved among species. Does this imply that the transcriptional interference could be a conserved mechanism actively used in higher eukaryotes?

Boundary activity established by transcriptional interference from SINE B2 repeat in mouse

The answer to this question comes from the recent analysis of mouse SINE B2 repeat in *Growth Hormone* (*GH*) gene locus [16••].

SINEs (short interspersed elements) are 75–500 bp long retrotransposon fossil which contain internal promoters for RNA polymerase III [47]. The promoters of almost all known families of SINEs are derived from *tRNA* (e.g. mammalian SINE B2) with two exceptions: promoters derived from *7SL RNA* (Alu repeats in human and SINE B1 in mouse) or *5S rRNA* (SINE3 in zebrafish genome). SINEs do not contain any protein-coding sequence and hijack LINE repeat encoded proteins for their transposition. These transposable elements are usually regarded as genomic parasites, with their fixed, often inactivated copies considered to be 'junk DNA'. Despite the abundance of SINEs in eukaryote genomes (e.g. constituting 14% and 8% of the human and mouse genomes, respectively) [48], it is unclear whether they are of benefit to the host genomes.

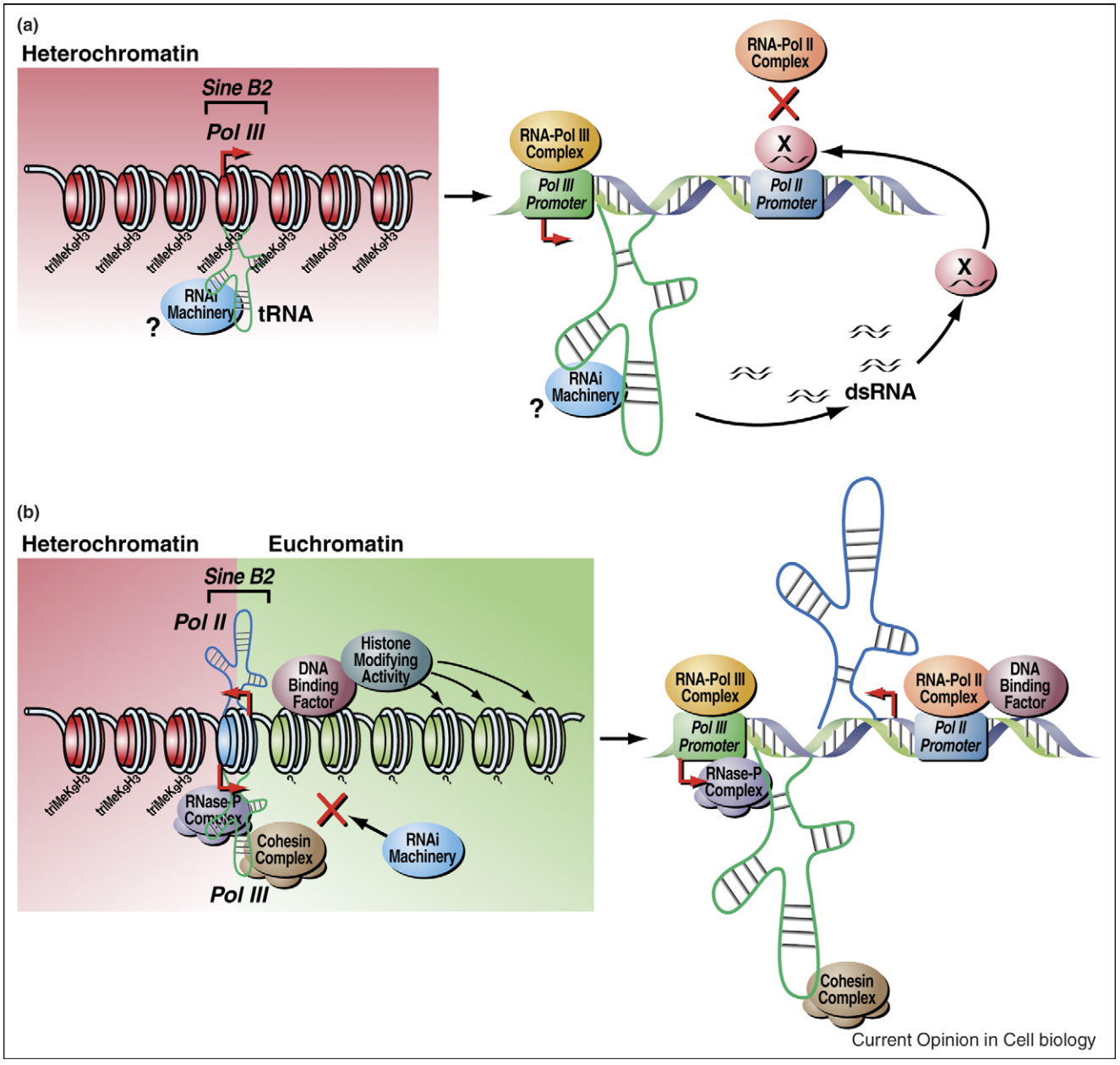
The B2 SINE family constitutes approximately 0.7% of total mouse genomic DNA [49]. An interesting feature of SINE B2 repeats is that in addition to Pol III promoter, SINE B2 contain an active Pol II promoter located outside the *tRNA* region [50•]. The 70-bp minimal Pol II promoter was initially delineated within SINE B2 allocated in the *Lama3* gene. This sequence has substantial nucleotide similarity within the B2 SINEs family. Moreover, Pol II activity of SINE B2 does not preclude the Pol III transcription originated in this *tRNA* portion of the repeat.

The data obtained from the functional analysis of the SINE B2 repeat in murine *GH* locus demonstrate that the repeat element is able to generate short, overlapping Pol II-driven and Pol III-driven transcripts. The striking difference in the transcriptional activity of this mouse *tRNA* pseudogene from the data on *tRNA* transcription reported in yeast system, is such that Pol II transcription from SINE B2 is activated in developmental and tissue-specific fashion and correlates with restructuring chromosomal domain for *GH* gene. Physical repositioning of *GH* gene locus from heterochromatin to euchromatic compartment was observed by FISH analysis within specific cell type. This repositioning coincides with Pol II transcriptional activity from the SINE B2 repeat and accompanied by changes in histone modification within the locus.

SINE B2 repeat within *GH* locus posses both context-independent insulator activity (based on enhancer-blocking analysis) and can buffer from spreading heterochromatic modifications from facultative heterochromatin flanking the murine *GH* locus at 5′. Therefore SINE B2 repeat can be viewed as a true genomic boundary. Promoter deletion/substitution analysis demonstrates that both Pol II and Pol III transcription is required to mediate the insulator function [16••]. Contrary to the mechanism suggested for the yeast system [42], the Pol II/Pol III transcriptional activation, which likely required assembly of a large multiprotein complex, does not generate a nucleosome-free region. The nucleosome encompassing the SINE B2 transcriptional unit still present in the area during the time when transcriptional activity of *tRNA* pseudogene can be recorded (VL, unpublished data).

How does Pol II gain access to sequences that are packaged as heterochromatin? There are several models which were proposed [51•] to explain mechanisms of heterochromatic transcription. One can argue that the promoters driving the transcription of SINE/Alu repeats, unlike the promoters of protein-coding euchromatic genes, have evolved to be somewhat impervious to heterochromatic repression and might be marked by epigenetic signature specifically dedicated to this occasion. Indeed, one strand of SINEB2 repeat in *GH* locus is always transcribed at a low level by Pol III transcriptional machinery even when the locus is heterochromatic. Can the formation of Pol III-mediated transcript from SINE B2 create a clamp in the Pol II

Figure 1



Hypothetical models for regulation of Pol II transcriptional activity within SINE B2 repeat. (a) RNAi-mediated processing of Pol III-transcript from SINE B2 can lead to the formation of the RNA/DNA clamp in the Pol II promoter portion of the repeat by engaging RNA-i molecular complexes, thus denying the recruitment of the Pol II or Pol II-recruiting factors to the site. (b) Recruitment of development-specific DNA binding factor(s) or their co-regulators to Pol II promoter can stimulate the repeat-associated change in histone modifications, resulting in dismissal of RNAi processing machinery. The Pol III transcript will not be processed in this case, no RNA/DNA clamp will be formed at the Pol II promoter, thus permitting access for Pol II machinery to SINE B2.

promotor portion of the repeat by engaging RNA-i molecular complexes, thus denying the recruitment of Pol II or Pol II-recruiting factors to the site? (Figure 1A) Could this restriction be lifted up by developmental-specific changes in histone modifications or by recruitment of developmental-specific DNA-binding factor (s) and their co-regulators? (Figure 1B) To date there is no experimental evidence that will provide the molecular basis for developmental-specific Pol II transcriptional activity of the SINE B2, though indirect evidence points to the situation that the B2 Pol II promoters can be bound and stimulated by the transcription factor USF (for upstream stimulatory factor), as shown by transient transfection experiments [50•].

tRNA, SINE/Alu transcription, and nuclear superstructure

It was known for years that newly transcribed Pol III-generated precursor transcripts of *tRNA* undergo processing and modifications through several steps, which include the removal of the 5′ leader sequence, elimination of the 3′ trailer sequence, splicing of introns in some *tRNA* isotypes, addition of CCA, and modification of nucleotides [52]. The steps of *tRNA* biogenesis are spatially and temporally ordered and regulated by the La protein [53,54], which has been shown to bind to chromatin of *tRNA* gene in Hela Cells [54]. Removal of the 5′ leader sequence of precursor *tRNA* is carried out by ribonuclease P (RNase P), a ubiquitous ribonucleoprotein endonuclease [55,56]. Eukaryotic RNase P ribonucleoproteins are large particles, when compared with their bacterial counterparts. The catalytically active form of human nuclear RNase P consists of an RNA component, termed H1 RNA, and at least 10 distinct protein subunits, designated Rpp14, Rpp20, Rpp21, Rpp25, Rpp29, Rpp30, Rpp38, Rpp40, hPop1, and hPop5 (see in [56]).

New studies demonstrate that the RNase P subunits are maximally bound to chromatin of *tRNA* and *5S rRNA* genes in dividing cells while dissociate from chromatin in mitotic cells [57•]. In contrast to *tRNA* and *5S rRNA* genes, the *7SL RNA* and possibly *U6 snRNA* genes seem not to be bound by RNase P subunits as determined by ChIP analysis. In fact, H1 RNA and its protein subunits are differentially concentrated in distinct intranuclear compartments — for example, nucleolus, nucleoplasm, and Cajal bodies [56], suggesting a possible differential sequestering of various families of transcribed SINE/Alu in the specialized nuclear compartments. Since these intranuclear compartments are known to be associated with specialized transcriptional activities and their structural integrity is dependent on continuous RNA synthesis, it is tempting to speculate that they might delineate SINE/Alu-mediated 'barrier body' in the way similar to 'insulator bodies' existence in Drosophila, thus providing not only separating the functional chromosomal domains (loop formation) but also mediating long-range interchromosomal and intrachromosomal interactions. The clustering of tRNA genes in specialized subnuclear locations has already been reported for *S. cerevisiae* [58] and formation of higher-order chromatin structure by this event might also partially explain why propagating a chromatin-remodeling signal beyond a *tRNA* gene is difficult, as it has observed in cases where *tRNA* genes serve as boundary elements for chromatin domains.

Whether Pol III or its transcription factors recruit RNase P on SINE B2 repeats awaits thorough analysis. It is highly possible that SINE/Alu repeats expression will be affected by RNAase P. It would be interesting to explore how this can effect barrier/insulator activity of the SINE B2 repeat or formation of higher-order nuclear organization. In addition, loading cohesin or cohesin-associated complexes may play a role in the repeat-mediated barrier formation of higher-order chromatin structure. A recently published studies in *S. cerevisiae* demonstrate that cohesion at *HMR* can be established by *tDNA*s that create silent chromatin boundaries [59,60••]. Furthermore, the experimental approach elaborated in this study indicates that recruitment of TFIIIB or a subsequent step in the RNA Pol III transcription pathway is required for the cohesion at *HMR*. These results, however, cannot distinguish whether cohesion relies on barrier activity *per se* or some upstream event, like transcription of the *tRNA* gene, required to generate barrier activity.

Why not to push beyond the 'classic boundaries'?

When it comes to viewing repeat-mediate barriers/insulators as a new subclass of true genomic boundaries, much work has to be done. New systematic approaches must be elaborated and our conceptual view on their role within the genomes should be re-evaluated. If these DNA elements play a role in establishing higher-order domains of chromosomal organization, then their activities might be modulated during both cell division and cell differentiation. If repeat-derived boundaries are regulatory elements, then how are they themselves regulated? Other factors than just Pol II and Pol III machineries which are directly involved in regulating their activities must be present in the nucleus. Such factors have not yet been identified or if they are defined how are they exploited by cells at specific loci under specific circumstances? What are the molecular steps involved in the recruitment of these factors and how does it correlate with the restriction of particular chromatin state? Is there a common mechanism or does the mechanism vary depending on the type DNA repeated sequence? Answer to these and many other questions will allow SINE/Alu repeated component of the genome to be exploited in research and medicine. But first, similar to the game of Cricket, lets define the rules and . . .Have a great game!

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