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Conformation Regulation of the X Chromosome Inactivation Center: A Model

Antonio Scialdone¹, Ilaria Cataudella², Mariano Barbieri³, Antonella Prisco⁴, Mario Nicodemi⁵∗

¹ Department of Computational and Systems Biology, John Innes Centre, Norwich, United Kingdom, ² Center for Models of Life, Niels Bohr Institute, Copenhagen, Denmark, ³ Dipartimento di Scienze Fisiche, Università di Napoli “Federico II,” INFN, Napoli, Italy, ⁴ CNR Istituto di Genetica e Biofisica “B. Traverso”, Napoli, Italy, ⁵ Dipartimento di Scienze Fisiche, Università di Napoli “Federico II,” INFN, CNR-SPIN, Napoli, Italy

Abstract

X-Chromosome Inactivation (XCI) is the process whereby one, randomly chosen X becomes transcriptionally silenced in female cells. XCI is governed by the Xic, a locus on the X encompassing an array of genes which interact with each other and with key molecular factors. The mechanism, though, establishing the fate of the X’s, and the corresponding alternative modifications of the Xic architecture, is still mysterious. In this study, by use of computer simulations, we explore the scenario where chromatin conformations emerge from its interaction with diffusing molecular factors. Our aim is to understand the physical mechanisms whereby stable, non-random conformations are established on the Xic’s, how complex architectural changes are reliably regulated, and how they lead to opposite structures on the two alleles. In particular, comparison against current experimental data indicates that a few key cis-regulatory regions orchestrate the organization of the Xic, and that two major molecular regulators are involved.

Introduction

X-Chromosome Inactivation (XCI) is the vital process occurring in female mammalian cells whereby one randomly selected X is transcriptionally silenced to balance dosage with respect to males [1–4]. XCI is regulated by a region on the X chromosome, the X inactivation center (Xic), which encompasses a key group of neighboring non-coding genes (see Fig. 1.A) including, e.g., Jpx, Xist, Tsix and Xite [1–4]. The fate of the X is determined by its Xist gene which is strongly upregulated on the future inactive X and repressed on the other X. In turn, Xist is negatively regulated by Xite/Tsix, and positively regulated by Jpx, Rnf12, and other factors [5–7].

Before random XCI starts, a complex epigenetic program, coupling transcription and chromatin remodelling [8,9] to pluripotency factors [10–12], produces a state where the Xic has the same spatial conformation on the two X chromosomes [13] and both Xist alleles are just weakly active. Upon XCI, an unknown symmetry breaking mechanism determines the opposite behaviour of the two Xist, and induces alternative modifications of the three-dimensional conformation of their Xic [13,14]. Finally, on the designated inactive X further chromatin reorganizations occur on the inactive Xist and Jpx alleles, and allows for alternative modifications of their Xic [13,14]. To understand the principles of chromatin organization, within the marine Xic case study, here we explore the scenario where chromatin conformations emerge from its interaction with diffusing molecular factors. We discuss general physical mechanisms whereby random Brownian molecules can: i) succeed in establishing stable, non-random conformations on the chromosomes; ii) reliably regulate specific conformational changes; and iii) produce opposite transformations on identical alleles exposed to the same environment (“symmetry breaking”). We investigate by computer simulations a schematic model consisting of two identical polymers which interact with a concentration of diffusing molecules [see Fig. 1.B]. In the light of current Xic 3C data [13], the model poses that along each polymer three types of regions exist type-α, β and γ) and predicts the existence of two types of regulatory molecules (type-A and B).

We show that the system thermodynamic stable states fall in distinct classes corresponding to different conformations. The polymers spontaneously select one of them according to molecule concentration/binding energy. Conformational changes are driven by thermodynamic phase transitions which act switch-like, regulated by given concentration/binding energy thresholds. The two polymers are exposed to the same environment, yet they can undergo alternative architectural modifications; we show that a symmetry breaking mechanisms is activated if the homotypic interaction between regulatory molecules rises above a threshold.

Comparison to experimental observations [1–5,13,21] suggests that the regions envisaged by the model can be approximately mapped along the Xic sequence as illustrated in Fig. 1.B, while type-A and B complexes could be related to an activating and a blocking regulator of Xist.
Author Summary

In mammalian female cells X-Chromosome Inactivation (XCI) is the vital process whereby one X, randomly chosen, is silenced to compensate dosage of X products with respect to males. XCI is governed by a region on the X, the X Inactivation Centre (Xic), which undergoes a sequence of conformational modifications during the process. The two Xic are exposed, though, to the same environment, and it is obscure how they attain different architectures. By use of computer simulations of a molecular model, here we individuate general physical mechanisms whereby random Brownian molecules can assemble chromatin stable architectures, reliably regulate conformational changes, and establish opposite transformations on identical alleles. In the case-study of the murine Xic, our analysis highlights the existence of a few key regulatory regions and molecular factors. It also predicts, e.g., the effects of genetic modifications in the locus, which are compared with current deletion/insertion experiments. The physical mechanisms we describe are rooted in thermodynamics and could be relevant well beyond XCI.

Model

We represent the relevant region of each X chromosome (see scheme in Fig. 1B) by a standard model of polymer physics, a self-avoiding bead chain [23]. In the light of Xic current 3C data [13], we pose that along each polymer there are, for simplicity, two type-\(\alpha\) regions which have an array of binding sites for type-A Brownian molecular factors. Each polymer has also two type-\(\beta\) regions with binding sites for a different kind of molecular factors (type-\(\beta\)). Finally, the polymers have a type-\(\gamma\) region whose binding sites can be bound by either type-A or B molecules. Thus, type-A molecules (resp. type-B) can bridge a type-\(\alpha\) (resp. type-\(\beta\)) and a type-\(\gamma\) site. For simplicity, with no loss of generality, we consider the case where the two types of molecules have the same concentration, \(c\), and the same affinity, \(E_{\alpha}\), for all binding regions. Similarly, we assume that type-\(\alpha\) and type-\(\beta\) regions have the same number of binding sites, \(n_0\), than type-\(\gamma\). The value of \(n_0\) is fixed to have a total binding site number of the order of known Xic binding molecules. As CTCF is a general chromatin organizer which has been associated to XCI and its Xic binding sites have been well characterized [17], we use it as an example (and set \(n_0=20\)). For simplicity, \(n_0\) is here also the length of the intervening inert sequences between them. Type-A (resp. type-B) molecules can bind, with multiple valency, each other with affinity \(E_{AA}\) (resp. \(E_{BB}\)); we set \(E_{AA}=E_{BB}=E_0\) and, considering the number of binding domains of CTCF, the valency to four.

We investigate by Monte Carlo (MC) simulations the conformations of the system as they spontaneously emerge when the three control parameters, \((c, E_{\alpha}, E_0)\), are varied. For computational purposes, the system lives in a cubic lattice with a lattice spacing \(d_0\), whose value corresponds to the typical size of a DNA binding site, and can be roughly estimated to be \(d_0\sim 10nm\). The volume concentration of molecules in our model, \(c\), can be related to molar concentrations \(\rho = c/d_0^3 N_A\), \(N_A\) being the Avogadro number (details in Text S1). Thus, for instance, a typical nuclear protein concentration of \(\rho \sim 0.1\mu\text{mol/litre}\) would correspond to \(c \sim 10^{-2}\%\). Below we consider concentrations in the range \(c \sim 10^{-4} - 10^{-6}\%\) and binding energies in the weak biochemical scale (a few units in \(k_BT\)). Finally, conversion of MC time unit to real time is obtained by imposing that the diffusion constant of our polymers is of the order of measured chromatin diffusion constants (see Text S1 for details).

Results

Establishing stable interactions

We first show that diffusing molecules can produce a looped conformation on each polymer where type-\(\alpha\) and type-\(\beta\) stably interact with type-\(\gamma\) region. The process is based on a thermodynamic mechanism (a phase transition, in the thermodynamic limit) which acts switch-like when concentration/affinity of binding molecules rise above a threshold [24].

Before describing our MC results in details, we illustrate the underlying mechanisms. A single, say, type A molecule forms a bridge between type-\(\alpha\) and type-\(\gamma\) regions via the stochastic double encounter of the molecule with its binding sites. This is, though, an unlikely event, especially if molecule concentration, \(c\) (or \(E_{\alpha}\), see below), is small. And the half-life of such a bridge is short when weak biochemical interactions are considered. Thus, on average the regions float away from each other (see pictorial representation in the bottom panel of Fig. 2, “Open State”). At higher \(c\) (or \(E_{\alpha}\)), however, many a molecule can bind type-\(\alpha\)-\(\gamma\) regions and stabilize the conformation via a positive feedback mechanism as their bridges reinforce each other and facilitate the formation of additional bridges. The concentration where such a positive feedback mechanism starts winning marks the threshold above which stable contacts are established (pictorial representation in the bottom panel of Fig. 2, “Stable Interaction”).

This pictorial scenario summarizes our MG results. For sake of simplicity, we consider first the case where molecule mutual interaction is turned off, \(E_0=0\), and set as initial configuration of
the polymers a randomly open conformation. We measure the interaction order parameter, \( \pi = (p_A + p_B)/2 \), where \( p_A \) (resp. \( p_B \)) is the probability to have, on a polymer, a contact of a type-\( \alpha \) (resp. type-\( \beta \)) with type-\( \gamma \) region. If neither type-\( \alpha \) nor type-\( \beta \) regions are in contact with \( \gamma \), the order parameter is zero, \( \pi = 0 \); if only one pair is stably interacting then \( \pi \) is close to 0, as no stable contact is statistically possible; instead, if \( c \) is high enough, \( \pi \) grows to a value close to one, \( \pi \approx 1 \), showing that both the type-\( \alpha \) and \( \beta \) loops are formed.

Conformation switch and sharp regulation

In the space of the control parameters, \((c,E_X)\), a sharp line separates the two regimes, as shown in Fig. 2 bottom panel: when \( c \) or \( E_X \) are small, contacts cannot be stable and \( \pi = 0 \); conversely, above the transition line the two loops conformation is reliably established on each polymer, and \( \pi = 1 \). Such a line marks the boundary between two thermodynamic phases [25]: it corresponds to the point where the entropy loss due to loop formation is compensated by the energy gain obtained from the establishment of the corresponding bridges.

The discovery of such a switch-like behaviour can also explain how loop formation can be sharply and reliably regulated in the cell by increasing the concentration of specific molecular mediators or the affinity to their DNA target sites, e.g., by chromatin or molecule modifications.

The position of the transition line is also dependent on the number of available binding sites, \( n_0 \), since, schematically, the overall binding energy scale is \( n_0 E_X \). Thus, non-linear threshold effects in genetic deletion/insertions of the locus exist.

Threshold values in real nuclei

From Monte Carlo results we can predict concentration (or energy) thresholds in real nuclei. For instance, in vivo measures of CTCF DNA binding energies give \( E_X = 20kT \), a typical value for TEs [26,27]: an extrapolation from Fig. 2 then predicts a threshold \( c_\pi \sim 10^{-3} \% \) corresponding to a typical nuclear protein molar concentration \( \rho \sim 10^{-2} \mu \text{ mole/litre} \) (see Text S1).

Finally, the mechanism leading to stable loop formation has to be fast enough to serve functional purposes. In our model we find that stable interactions are established on scales of the order of minutes (see Fig. 2 top panel and Text S1), a range consistent with biological expectations.

Symmetry Breaking mechanism

The mechanism to induce conformational changes illustrated above acts “symmetrically” on the two polymers. Now we show that molecule homotypic interaction, \( E_0 \), can break the polymer symmetry via a different thermodynamic mechanism. More precisely, if \( E_0 \) (and \( c \), see below) is above a critical threshold, a single major aggregate of type \( A \) molecules and a single one of type \( B \) are formed because of homotypic binding cooperativity; in facts, the energy gain in forming a single cluster of \( A/B \) molecules (which maximizes the number of possible chemical bonds) compensates, if \( E_0 \) is large enough, the corresponding entropy reduction. The single, say, type-\( A \) aggregate will then randomly bind just one polymer, leaving the other one “naked” (pictorial representation in the bottom panel of Fig. 3, “Symmetry Breaking”).

Type-\( A \) and \( B \) aggregates bind opposite polymers because \( A \) and \( B \) molecules compete for binding sites in the type-\( \gamma \) region. Hence, if a fluctuation increases the presence of, say, \( A \) molecules on one polymer, cooperativity tends to favor their assembling at that site and \( B \) molecules are expelled; in turn, the depletion of \( A \) around the other polymer favors the assembling of \( B \) molecules on it. On the polymer where the \( A \) cluster binds the type-\( \gamma \) region, the \( B \)-related loci can no longer be stably linked, and their loop opens; the opposite situation happens on the other polymer.

The above scenario results from our MC simulations. We measured the symmetry breaking order parameter, \( m_A = (p_A^0 - p_A^1)/(p_A^0 + p_A^1) \), where \( p_A^0 \) is the average local concentration of \( A \) molecules around the type-\( \gamma \) region of polymer \( i = 1,2 \). The \( m_A \) parameter is close to zero if an equal amount of \( A \) molecules is present around the two polymers, whereas it approaches one if the symmetry is spontaneously broken (|\( m_A \)| and...
m = (mA + mB)/2 (behave analogously). Fig. 3 top panel shows the time evolution of mA(t) from an initial configuration corresponding to the symmetric state (schematic picture in the bottom panel of Fig. 3, “Stable Interaction”) where each polymer has two stable loops as seen before: if E0 is small, mA remains close to zero at all times and the system remains in a symmetric state; conversely, if E0 is high enough, mA approaches one because A molecules reside mostly around just one, randomly chosen polymer and the symmetry is broken (schematic picture in the bottom panel of Fig. 3, “Symmetry Breaking”). The phase diagram of Fig. 3 bottom panel shows that the symmetry breaking mechanism is switch-like too: in the (c, E0) space, as soon as a narrow transition line is crossed the system switches from a symmetrical polymer state to a broken polymer symmetry state. More details are in the Text S1.

For sake of simplicity, we considered the case where the concentration/DNA affinity of molecules A and B are the same. However, such an assumption does not affect our general results. The only condition for the Symmetry Breaking and Configuration Switch mechanisms to be triggered is that concentration/interaction energy of both types of molecules rise above the appropriate threshold.

**Symmetry Breaking in real nuclei**

As far as XCI is concerned, the predicted single B molecule aggregate is interpreted as an Xist repressing factor (a Blocking Factor, BF) and designates the future active X. The A aggregate marks the X where Xist transcription is enhanced and is interpreted as an activating factor (AF). Importantly, the thresholds predicted by our theory for the symmetry breaking mechanisms also fall in the correct biochemical range (see above and Fig. 3 bottom panel).

The time scale required to break the symmetry in a real nucleus can depend on a number of details. Our MC provides, thus, only a very rough order of magnitude estimate. As shown in Fig. 3 top panel, such a time scale is predicted to be around 10 hours, a value of the order of the time required for XCI initiation.

In males other processes could intervene, yet it is easy to see how the same two factors mechanism can work, i.e., why the only X is usually bound by the B aggregate (and not by A) to repress Xist. In fact, the affinities of A and B molecules for the type-γ region are expected, in general, to be different: EX > EX, . Hence, if EX is larger than E, it is thermodynamically convenient that B molecules bind the X, a difference of a few units in kT being sufficient to skew of orders of magnitudes the binding probability of A and B.

Finally, variants of the model can be considered to account for further biological details. For instance, additional molecular factors, or the effects on polymer colocalization can be discussed (see Text S1), but no relevant changes to the present scenario are found.

**Discussion**

Our schematic model (Fig. 1.B) predicts that two kinds of molecular regulators, type-Å and B molecules, interact with a set of specific regions along the polymers. Current 3C data [13] suggest that our type-α and type-β regions map respectively in the area 5’ and 3’ to Xist, while the type-γ region is in between.

We showed that in our model only three classes of stable conformational states exist (see Fig. 4 A,B,C). The system spontaneously falls in one of them, according to molecule concentration and homotypic interaction, c and E0. State changes are regulated by a “conformation” and by a “symmetry breaking” switch, related to two distinct thermodynamic phase transitions [25]. The switches are controlled by changing c and E0 above/below specific threshold values. Their on/off nature can explain how a sharp regulation of nuclear architecture and stochastic choice of fate can be reliably obtained by simple strategies, such as protein upregulation or chromatin modification. Importantly, the model predicts energy/concentration thresholds which are in the expected biological range (weak biochemical energies, fractions of μmole/litre concentrations).
We now discuss how the present scenario can recapitulate in a unified framework important experimental results on XCI.

Xic architecture, “counting” and “choice”

Before XCI, the Xic conformation is found to be identical on the two X’s [13]: Tsix and Xite genes are looped onto a “buffer” region; similarly, Jpx, Xist and the “buffer” form a second hub with Xist. Upon XCI, on the future active X, the Jpx-Xist-buffer hub opens while Xite remains in contact with Tsix. On the other X, instead, the Tsix-Xite interactions is lost whereas Xist and Jpx remain in contact.

Our model rationalizes how those elements are sharply regulated to recognize each other and to form stable interactions based on weak biochemical bonds. It can also explain how the same physical elements later at XCI spontaneously break the X symmetry. The molecular aggregate bound, in our model, to the type-b regions (which should encompass the Tsix-Xite area) is interpreted as a factor related to Xist silencing (i.e., to its Blocking Factor, BF [1,2,4]) and designates the future active X; the different aggregate bound to type-a regions, encompassing the Jpx area of the other X would be linked to an Xist activating factor (AF) [5,6,22]. The link between architectural changes and choice of fate emerges here naturally.

During XCI establishment, the inactive X undergoes further architectural reorganization [3,14,15]. The mechanistic details of those conformational changes are still not understood, but they could involve mechanisms as those illustrated here.

Other interesting models have been proposed for “counting-choice” at XCI, but still none had focused on the Xic spatial organization, including our original Symmetry Breaking theory [19]. In the approach of ref. [21], each X chromosome is assumed to have an independent probability to initiate inactivation. Two competing factors exist: an X-linked XCI-activator and an XCI-inhibitor produced by autosomes. In a male XY cell the XCI-activator concentration is too low to initiate the inactivation of the only X; in female XX cells the initial XCI-activator concentration is, instead, above the threshold needed to start XCI. As soon as one X is inactivated, the XCI-activator concentration falls down to the levels found in males, and thus the other X remains active. A different model [22] poses that two types of sites are present on the X: “XCI-init” which is responsible for the initiation of inactivation of the X bearing it, and “XCI-repres” sites which inhibit the action of “XCI-init”. Each active X produces molecules, say A molecules, which bind to some autosomal sites. If these sites are saturated, the autosomes produce a set of molecules I, which, with a “Symmetry Breaking” mechanism [19], self-assemble into a single molecular factor and inhibit the activity of “XCI-repres” sites on one of the two X, determining its inactivation. As the availability of the A signal is reduced, it is no longer sufficient to saturate the autosomal receptors, and the remaining X remains active.

Figure 4. System states and transitions. The figure summarizes the system possible states and how they change by action of the Conformation and the Symmetry Breaking switch (top pictures are from MC simulations, bottom ones are schematic drawings). The switches have a thermodynamic nature and are regulated by increasing, e.g., c and E0 (i.e., molecule concentration and homotypic interaction) above precise threshold values, c_tr and E_trSB. A) For c < c_tr and E0 < E_trSB, the polymers are found in a random open state. B) For c > c_tr, a conformation change is activated: type-a and type-b regions stably interact with type-c, and a two loop conformation is established symmetrically on the two polymers. C) If E0 > E_trSB, a symmetry breaking occurs as the type-a loop persists on one, randomly selected, polymer (where type-b loop is released), whereas the other polymer takes the opposite conformation. This results from the self-assembling of a single major aggregate of type-A and of type-B molecules competing to bind to type-c region.

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The mechanisms for conformational changes we discussed here are rooted in thermodynamics and are, thus, very robust to differences in molecular details. They could apply then to all the mentioned models for “counting&choice”. An interesting question concerns the applicability of those models to mammals other than mice. Important differences have emerged, for instance, between human and mice XCI [28,29]. As stated above, the mechanisms we discussed for Xic architecture in mouse stem cells are very robust, yet data on other organisms are still too scarce to decide whether such mechanisms might apply elsewhere.

**Xic deletions/insertions and XCI**

The phenotype of key deletions along the Xic (see reviews in [1,2,4,19,22] and refs therein) can be explained by our model. The Δ65kb deletion [30] removes 65kb encompassing Xite and part of Xist/Tsix. In heterozygous females the deleted X is always inactivated. In males it leads to the inactivation of the only X; the shorter the deletion considered within the Δ65kb (see ΔA5, ΔAJ, Δ4V, Δ34 [31]), the smaller the fraction of ectopic X inactivations in a population.

Those deletions, in our model, map into sites where the Xist “blocking factor” (BF) binds (and blocks inactivation of that X): Δ65kb removes a large portion of binding sites, thus the deleted X has a strongly reduced affinity for the BF (w.r.t. the wild type X) which does not bind there; the shorter the deletion, the weaker the effect. So, in heterozygously deleted females a skewed random XCI occurs, whereas in males the only X can be inactivated. These deletions can also impact the formation of the BF itself because the involved regions possibly encode some of its components.

Heterozygous TsixΔAG [32] and XiteΔL [33] deletions in females also result in the inactivation of the deleted X. Their homozygous counterpart produces, though, “aberrant counting/chaotic choice”, i.e., presence of two active or inactive X’s in a fraction of the cell population [18]. While that cannot be easily rationalized by other models (see, e.g., [21]), in our framework it is originated simply because the BF can fail to bind at all [34].

ΔXTX is deletion including Xist, Tsix and Xite, which in heterozygous causes a skewed XCI, as only the Wild Type X gets inactivated [21]. In the frame of our model ΔXTX could have a double effect: on the one hand, it hinders the binding of the AF and BF to the deleted X, by removing a number of their binding sites; on the other it affects especially the BF, since it removes the Tsix/Xite genes which are presumably linked to some of the BF components. Thus, the overall effect will be that while the deleted X remains active (as it lacks Xist), the BF is depleted and the AF wins the competition for binding the Wild Type chromosome, which is then inactivated.

Transgenic insertions are also interesting [35]. One of the predictions of our model is the highly non-linear effect of deletion/insertion, due to the “switch-like” nature of the underlying thermodynamic mechanism. The insertion experiments of ref. [35] support this view: long Xic transgenes can cause inactivation on male ES cells only when they are present in multiple copies, while single insertions do not have appreciable effects. The outcome of other deletions/insertions, such as XistΔpromoter [36], XistΔ1-5 [37], Jpx [5], Rnf12 [6], etc., are similarly explained (see Text S1). XCI in diploid cells with more than two X and in polyploid cells [21] can be understood as well in our scenario (see Text S1), but additional biological hypotheses are required, since key pieces of information are still missing.

In summary, we illustrated physical switch-like mechanisms establishing conformational changes and symmetry breaking in a polymer model. For clarity, we included just the required minimal ingredients, but our model can accommodate more realistic molecular details. It can be mapped into the Xic region of X chromosomes to explain their complex self-organization and other important aspects of random XCI, such as the deep connection between Xic architectural changes and Xist choice of fate, reconciling within a single framework a variety of experimental evidences. The on-off character of the underlying mechanisms can also explain how sharp and reliable regulation of XCI can be attained by simple strategies, such as gene upregulation or chromatin modification.

It supports a picture where random XCI could be governed by a few core molecular elements and basic physical processes. Two main groups of molecular factors are envisaged to control the process and to produce an activating and a blocking factor for Xist. The specific polymer regions in our model emerge as key cis-regulators which orchestrate functional contacts along the Xic. Experiments targeted at that area could test their role. The model also predicts threshold effects of, e.g., genetic deletions of the regulatory regions.

The precise nature of factors and sequences involved at XCI could differ from the minimal one considered here, yet the thermodynamic mechanisms we discussed are robust and independent of the specific molecular details. Similar mechanisms could be, thus, relevant to XCI and, more generally, to other nuclear processes requiring, for example, chromatin spatial reorganizations [38–40] or alternative choices [41].

**Supporting Information**

**Text S1** Supplementary text and figures covering the following topics: additional details on the model, polymer colocalization, effects of Xic Deletion/Insertion experiments on XCI and XCI process in cells with more than two X’s. (PDF)

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**Author Contributions**

Conceived and designed the experiments: AS, IC, AP, MN. Performed the experiments: AS IC AP MN. Contributed reagents/materials/analysis tools: AS IC MB. Wrote the paper: AS MN.

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