Published in final edited form as:
Curr Opin Genet Dev. 2009 December ; 19(6): 550–556. doi:10.1016/j.gde.2009.10.004.

The structure-function link of compensated chromatin in Drosophila

John C. Lucchesi
Department of Biology, Emory University, 1510 Clifton Road, Atlanta, GA 30322

John C. Lucchesi: lucchesi@biology.emory.edu

Summary

All aspects of transcription are controlled by complexes that modify or remodel chromatin at the level of individual genes, gene clusters, or whole chromosomes. The MSL complex that is responsible for dosage compensation in Drosophila is an example of complexes that operate at the whole-chromosome level on the transcription of individual genes. Recent experiments using traditional genetic analysis, molecular cytology, chromatin immunoprecipitation, or microarray technology have characterized the function of the two known enzymatic components of the MSL core complex and have identified the sequence characteristics that allow spreading of the complex along the X chromosome and a specific histone modification of active X-linked genes to which it is attracted. Further progress in understanding the function of this complex will benefit from biophysical approaches.

Introduction

Dosage compensation refers to the equalization of most X-linked gene products between males and females. In Drosophila, it is mediated by the MSL complex consisting of a core of five proteins, as well as one of two non-coding RNAs. The complex preferentially associates with numerous sites on the X chromosome in somatic cells of males but not of females. It is responsible for an enhancement of the transcriptional rate of a substantial number of X-linked genes, thereby mediating a compensatory effect for the difference in dosage of these genes between males and females [1]. Although all of the genes that encode the MSL complex protein subunits are transcribed in females, the complex is absent because the SXL protein that is responsible for female differentiation prevents the translation of the *msl2* gene transcript [2, 3].

The presence of the MSL complex on the male X chromosome is correlated with a significant increase of histone H4 acetylated at lysine 16 (H4K16ac; [4]). This acetylation is the result of the activity of MOF - a histone acetyltransferase of the MYST family [5]. In order to display maximal activity and strict specificity, MOF must be included in the MSL complex [6] and, in particular must associate with the MSL1 and MSL3 subunits [7]. In addition to this enzyme, the MSL complex of Drosophila includes an ATP-dependent DEXH-box RNA/DNA helicase (MLE) that prefers double-stranded RNA or RNA/DNA hybrid substrates with a short 3′ overhang [8].

In Drosophila males, the complex is believed to assemble at the locus of the two *roX* genes and then spread to numerous additional sites along the X chromosome for which it has a complete range of affinity levels [9,10]. Approximately 40 of these sites were defined as "high-affinity" because a partial complex can bind to them [11]. The nature of these sites was poorly understood until Gilfillan *et al*. [12•] identified a core sequence and proposed that dispersed along the X chromosome are clusters of several distinct but degenerated sequence motifs for which the MSL complex exhibits a complete range of affinity. Recently, using immunoprecipitation and microarray hybridization or sequencing, from 130 to 150 X-chromosome sites predominantly enriched for a GA repeated sequence for which the MSL complex has particular affinity have been identified [13••,14••].

To define the role of a chromatin remodeling complex on gene function it is necessary to develop a detailed understanding of its association with target genes and of the mechanism that it uses to modulate the transcription of these genes not only at the molecular but also at the biophysical level. The purpose of this review is to set the stage for the study of the function of the MSL complex at the level of chromatin architecture.

Spreading of the MSL complex along the X chromosome

The generally accepted model has the MSL complex initially associating with chromatin entry sites and subsequently accessing active genes in order to enhance their transcription (Figure 1). Spreading beyond the 50 or so high-affinity sites to the numerous, newly characterized entry sites requires the complex to be fully assembled [15] and for its two enzymatic components - MOF and MLE - to be functional [16]. The mutation in MOF that prevents spreading is in the acetyl-CoA binding site and it is not clear whether the effect on spreading is caused by the loss of acetyltransferase function or by a conformational change in the protein, due to its failure to bind the co-enzyme, that in turn affects the general conformation of the complex.

MLE is related to the ATPases present in complexes that remodel chromatin by altering the positioning or the architectural relationship between histone octamers and DNA [17]. In contrast to MLE none of these enzymatic subunits have been shown to possess double-stranded nucleic acid unwinding activity. To determine whether MLE has been subsumed by the MSL complex just for its ATPase function or for its ATP-dependent helicase activity, we analyzed mutations that allow MLE proteins to retain the ATPase but not the helicase activity. MSL complexes containing these mutant proteins could not spread along polytene chromosomes beyond the high-affinity sites but enhanced the transcription of genes immediately adjacent to these sites [18••]. These observations clearly indicate that the ATPase activity is required for MLE's role in the transcriptional enhancement of a targeted gene while the helicase activity is necessary for the spreading of the complex along the X chromosome.

To date nothing is known of the *in vivo* substrate of MLE or of the mechanistic basis of its function in dosage compensation. A biophysical approach using single-molecule microscopy assays based on tethered particle motion and magnetic tweezers techniques [19] could determine whether MLE is capable of altering the torsional stress of DNA molecules or of translocating along a nucleic acid substrate.

Targeting genes for dosage compensation

Several years ago, we showed that the MSL complex is attracted to activated genes [20]. The questions that remained were: (1) What attracts the MSL complex to a transcribing gene? (2) How does the complex manage to acetylate H4K16 throughout the transcriptional unit?

Furuhashi *et al*. [21•] used RNAi in flies to knock down supercoiling factor (SCF) - a protein that generates negative supercoils in DNA in conjunction with topoisomerase II. They noted a lethal effect in males more pronounced than in females. They showed that depletion of SCF inhibits dosage compensation of X-linked genes but, surprisingly, does not prevent the complex from an apparently normal distribution along the X chromosome in salivary gland nuclei. Interestingly, in Drosophila SCF is present predominantly at promoter regions (Susumu Hirose, personal communication) where it may facilitate the loading of the MSL complex onto active genes. From the promoter region, the MSL complex would proceed towards the 3′ end, perhaps in association with the transcribing RNA polymerases II.

An alternative, possibility is that the complex associates with the nucleosomes of a transcribed region in an opportunistic manner, for example between successive elongating polymerases and from whatever neighboring locus where it may be concentrated (upstream or downstream of the transcribing gene). The rationale for this possibility is as follows. The presence of the MSL complex is increased at the 3′ end of transcriptional units [22,23] as we had predicted by mapping the level of H4K16ac [24]. In contrast, following activation, the density of RNAPII on most actively transcribing genes remains skewed towards the 5′ end; this may interfere with the association of the MSL complex in this region of X-linked genes. In the body and towards the 3′ end of genes, elongating polymerases are relatively more sparsely spaced and there may be a greater opportunity for complex association. In addition, we had shown that when the MSL complex is recruited to a GAL4-induced promoter construct inserted in a region of the X chromosome normally deprived of the complex, the immunofluorescence signals generated by the GAL4 and MSL antisera do not overlap. In all cases where the site of insertion had been sequenced, the MSL protein was in a downstream position, relative to the GAL4 protein. These observations had led us to conclude that the MSL complex is localized on the transcribed portion of the region activated by the inserted promoter [20]. Evidence for this contention was provided by Larschan et al. [25•] who reported that the absence of H3K36me3, a mark of active transcriptional units, while lethal to both females and males leads to a reproducible reduction in the level of MSL binding in males and to a concomitant change in transcript levels of MSL target genes. They suggest that recognition of H3K36me3 is one contributing factor to MSL complex targeting that involves additional features of transcribed genes.

What is the function of the H4K16 acetylation mediated by MOF?

The undisputed correlation between enrichment in H4K16ac in the X chromosome and the enhanced transcription of X-linked genes in males has been known for a number of years. As is true in regard to most epigenetic events described to date, the mechanistic link between this particular histone modification and its effect on transcription – the link between cause and effect – was a proverbial black box. The basis for beginning to understand its content is Karoline Luger's seminal description of the structure of the nucleosome (Figure 2) and of a probable inter-nucleosomal interaction involving the acidic patch formed by an H2A/H2B dimer in one nucleosome and the basic tail of histone H4 from a neighboring nucleosome [26,27]. These observations led us to the hypothesis that the acetylation of lysine 16 on the tail of histone H4 lessens inter-nucleosomal interactions thereby facilitating nucleosomal eviction by the RNAPII complex, thereby enhancing the rate of elongation [24]. In support of this hypothesis, Dorigo *et al*. [28] first reported the effect of histone tails on electrostatic interactions between neighboring nucleosomes. More recently, the acetylation of H4 at lysine 16 was shown to prevent the conversion of reconstituted nucleosomal arrays into 30 nanometer fibers that are thought to represent a level of compaction of native chromatin unfavorable to transcription [29••].

The very act of transcription is associated with alterations in the chromatin organization of transcriptional units. Are there features of chromatin reorganization that are uniquely

associated with the enhanced level of gene expression mediated by the MSL complex? This question could be addressed by determining differences in restriction enzyme sites accessibility and nucleosome positioning on compensated and control genes. Unfortunately, given the average transcription enhancement of two-fold, these approaches may not yield meaningful signal to noise ratios. In contrast, the faithful reproduction of dosage compensation of a reporter gene carried on a plasmid introduced into S2 cultured cells by transfection [30••] provides the opportunity to investigate the effect of H4K16 acetylation on chromatin structure by probing for topological changes (Box 1). The type of results that can be obtained are illustrated in Figure 3.

Does the MSL complex act by reducing the level of pausing of RNAPII on X-linked genes?

Since the seminal observation by John Lis that uninduced heat shock genes carry a transcriptionally engaged polymerase that has stalled after synthesizing a very short RNA transcript [31] evidence has accumulated that numerous genes in the genome display paused polymerases [32–34]. Surprisingly, a number of active genes have a greater concentration of RNAPII in their promoter region than within their body indicating that pausing is not just used to shut off uninduced genes but is also used to regulate the transcriptional output of active genes. These observations lead to the hypothesis that the chromatin modifications mediated by the MSL complex may exert their effect on transcription by reducing the pause of X-linked genes thereby increasing the number of polymerases that are engaged in elongation.

A characteristic of gene promoters with stalled or pausing polymerases is a significant enrichment in GAGA factor binding sites [35]. The GAGA factor exerts both positive and negative effects on gene transcription by facilitating chromatin remodeling and maintaining promoters in a conformation accessible to other regulatory factors. A reduction in the level of this factor affects the viability of males to a greater extent than females [36]. In addition, the small number of autosomal sites that are normally targeted by the MSL complex (5 to 7) is approximately doubled. These effects could be simply a reflection of the general role played by the GAGA factor on transcription: the necessity for males to hyper-transcribe their X-linked genes would render them more sensitive to general transcription disturbances than females. Yet, the similarity of the GAGA factor binding sites with the GA-rich X-chromosome binding sites of the MSL complex reopens the question of a functional role for this factor in dosage compensation [13••,14••].

Interactions of the MSL complex with general chromatin assembly and nucleosome positioning complexes

The X chromosome in males responds dramatically to the loss-of-function of the general chromatin assembly complexes ACF and CHRAC and the nucleosome repositioning complex NURF. *In vitro*, ACF and CHRAC establish regularly ordered arrays of nucleosomes [37,38] while NURF disrupts nucleosome periodicity [39]. Loss-of-function mutations in ISWI, the ATPase common to all three complexes, or in a subunit unique to the NURF complex [40] transform the male X chromosome in salivary gland polytene chromosome preparations into a chromatin mass that has lost all morphological features. X-chromosome morphology can be rescued in males by preventing the occurrence of H4K16ac. In vitro, the interaction of purified ISWI with nucleosomes is abrogated if H4 is acetylated at lysine16 [41]. Recently, Bai *et al*. [42] reported that in a mutant *nurf* background, loss-of-function mutations in either *roX1* or *roX2* lead to a more normal appearance of the polytenic X in the general region of the mutation; conversely, a wild type *roX* transgene relocated to an ectopic autosomal location nucleated a region of disorganization at its site of insertion. These authors also provided evidence that the

NURF complex inhibits the synthesis of the roX RNAs in wild type females and prevents the over-transcription of *roX2* in males. Grau *et al*. [43] have reported that mutations in the *dAda3* gene cause a defect in the banding organization of polytene chromosomes in both males and females; once again, the X chromosome in males is more severely affected. Finally, Carre *et al*. [44] have shown that mutations in the histone acetyl transferase Gcn5 and the ATAC complex component Ada2a induce a specific decondensation of the X chromosome in mutant males. In contrast, the Drosophila RSF (remodeling and spacing factor) complex consisting of dRsf1 and ISWI does not affect the appearance of polytene chromosomes in either sex [45].

The MSL complex also interacts specifically with structural heterochromatin components Su (var)3–7 and Su(var)2–5/HP1, with the histone H3K9-specific methyltransferase Su(var)3–9 and with the tandem kinase JIL-1. Over-expression of Su(var)3–7 results in morphological effects in the larval salivary gland polytene chromosomes of both males and females, but the male X is most affected as it assumes a very small and highly compacted shape [46]. Loss of this heterochromatin protein or loss of HP1 results in a polytene X chromosome phenotype in males that is similar to the one induced by ISWI knockdown [47]. In their most recent paper, Spierer et al. [48••] report that the distribution of the MSL complex is abnormal on male polytene X chromosomes when the latter are enriched in heterochromatin by over-expression of Su(var)3–7. JIL-1 localizes to all polytene chromosomes but is substantially more abundant on the X chromosome in males [49]. Loss-of-function alleles result in global changes in the morphology of polytene chromosomes: the X chromosomes of females and the autosomes of both sexes exhibit some abnormal coiling, while the male X is once again shorter, fatter and without any evidence of banding [50].

The general similarity of the effects of very different complexes, remodeling activities or structural proteins strongly suggests that the common denominator may be the unique characteristic of the chromatin of the male X chromosome which renders it more sensitive to disturbances than autosomes or X chromosomes in females. These effects have been described by means of genetic and cytological experiments; their molecular basis should be sought at the biophysical level, using reconstituted chromatin fibers and approaches pioneered by Jeffrey Hansen and used effectively, for example, by Shogren-Knaak *et al*. [29].

Conclusions

In this review, I have highlighted the progress that has been made recently in understanding the function of the two known enzymatic components of the MSL complex, as well as in characterizing some of the parameters that allow the complex to spread along the X chromosome. A complete definition of these features and of the mechanism used by the complex to achieve transcriptional enhancement will rely increasingly on biophysical approaches.

Box 1

Topoisomers distribution

The wrapping of DNA around a histone octamer, induces one negative supercoil in the DNA molecule (a linking number change of −1) that is protected from relaxation by topoisomerase I. As the plasmid is extracted, endogenous topoisomerase I activity relaxes the linker DNA between nucleosome cores and the number of negative supercoils that remain is an indication of the level of protection conferred to the DNA by its association with nucleosomes. The difference in linking number of two plasmids of equal size can be resolved by either one-dimensional or two-dimensional electrophoresis in the presence of chloroquine. Binding of chloroquine unwinds the DNA helix and results in the loss of negative supercoils. At different chloroquine concentrations, each plasmid can be resolved

as a normal distribution of topoisomers whose center reflects the plasmid's linking number. A chloroquine concentration that gives the best distribution of topoisomers for both plasmids is chosen to carry out the linking number comparison.

Acknowledgments

Supported by grant GM15691 from the National Institutes of Health.

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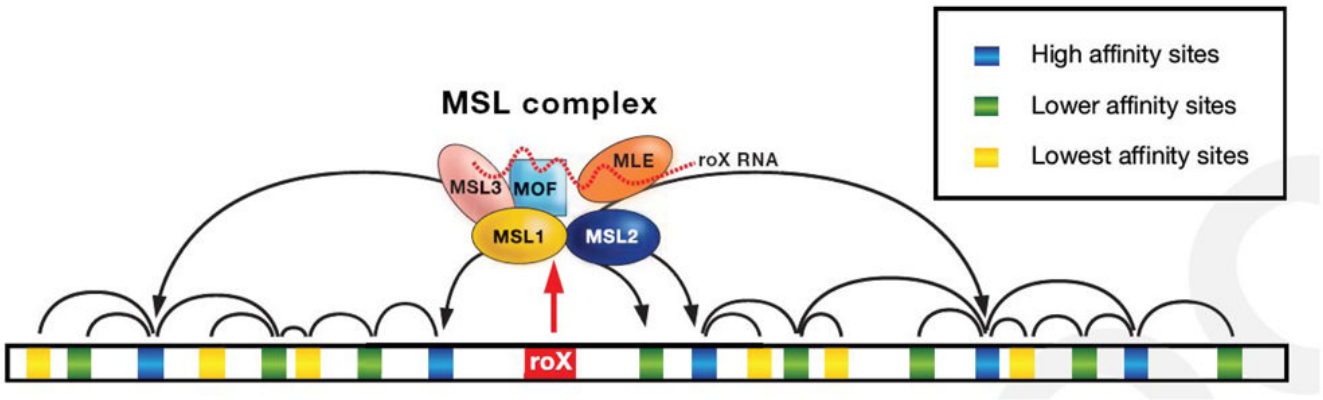

Figure 1. Spreading of the MSL complex along the X chromosome

The MSL complex assembles at the loci of the two roX genes that are located on the X chromosome. This is necessary because the roX RNAs are unstable unless they associate with some of the protein subunits of the complex. Assembled complexes then access the many sites along the X chromosome for which they have different levels of affinity. Ultimately the MSL complexes associate with those X-linked genes whose transcription is thereby enhanced.

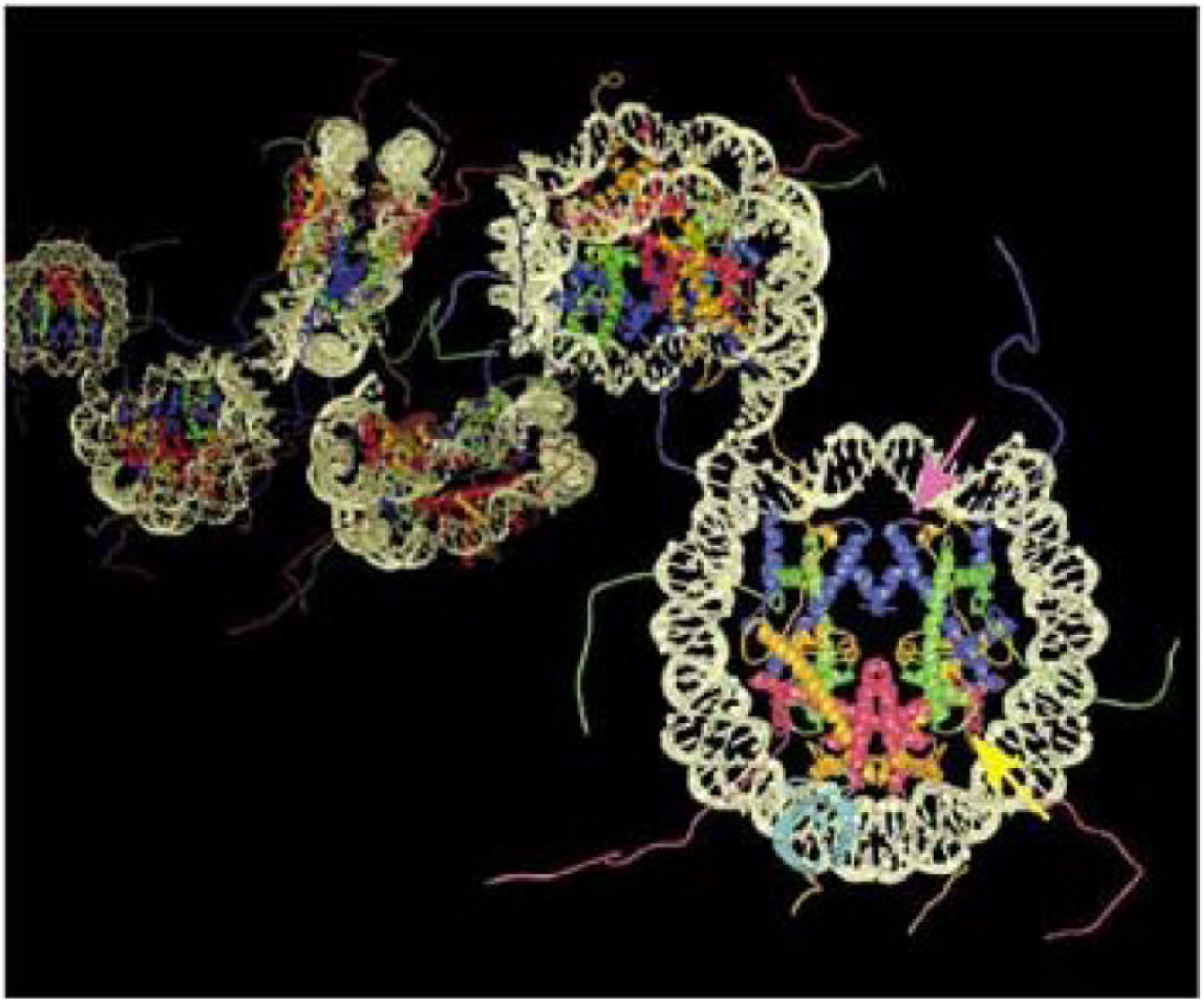

Figure 2. Structure of a chromatin fiber

The interaction of neighboring nucleosomes via the N-termilal tails of their core histones can be modified by covalent modifications such as the acetylation of histone H4 at lysine 16 (Reprinted with permission [51]).

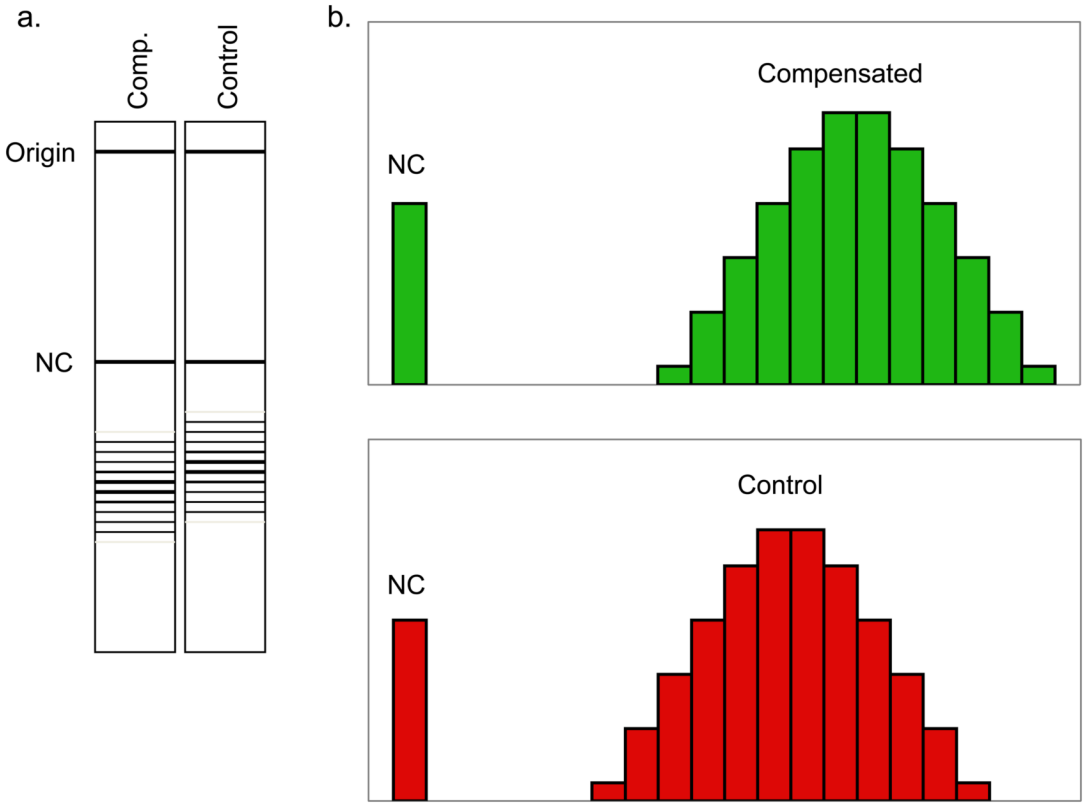

Figure 3. Example of a topological analysis of a plasmid subjected to dosage compensation

(a) Topoisomers are resolved in a chloroquine-containing gel. **(b)** Histogram representation of the topoisomers. Upper panel corresponds to the compensated plasmid; lower panel is a non-compensated control plasmid. The height of a bar represents the intensity of the corresponding bands in the gel. NC is the position of the nicked plasmid in the gel. The difference in the relative position of the two topoisomer distributions indicates a difference in the nucleosomal organization in the two plasmids.