Chromosome structure inside the nucleus

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Recent *in situ* three-dimensional structural studies have provided a new model for the 30 nm chromatin fiber. In addition, research during the past year has revealed some of the molecular complexity of non-histone chromosomal proteins. Still to come is the unification of molecular insights with chromosomal architecture.

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Introduction

In some unknown way, the massive length of nuclear DNA is organized into chromatin and chromosomes competent for transcription, replication and mitosis. There is no single chromatin or chromosome structure. Instead, it is likely that there is a set of interconvertible states dependent on DNA–nucleoprotein interactions and regulated by the cell as it passes through the cell cycle. Many recent reviews have focused on the role of chromatin structure, especially at the level of the nucleosome, in transcription (see Croston and Kadonaga, this issue, pp 417–423) [1–5]. Because of limited space, we have focused on recent advances in the determination of the structure of chromatin by electron microscopic (EM) tomography and two classes of proteins as examples of molecular regulation of chromatin and chromosome structure.

The 30 nm fiber structure

The winding of naked DNA around histone octamers forms an 11 nm 'string' of nucleosomes connected by free DNA. Under certain ionic conditions, or in the presence of histone H1, this fiber folds into a 25–30 nm chromatin fiber [6]. This structure, mediated *in vivo* by H1, may help repress transcription by making DNA sequences inaccessible to transcription factors [7]. Most studies of the 30 nm fiber structure have relied on nuclease digestion of interphase nuclei to release chromatin fragments. Data from such studies have led to numerous models for the 30 nm fiber, most of which predict some form of helical symmetry [6]. For many years, determination of the correct model has seemed an intractable problem. To add to the uncertainty, three-dimensional EM tomographic reconstructions of isolated, negatively stained fibers failed to reveal helically ordered arrangements, yet it was not possible to determine the nature of the actual organization [8•].

Recent research has led to significant advances. The problem of structural rearrangements during fiber isolation [9] was circumvented by three-dimensional reconstructions of fibers preserved *in situ* in the nuclei of starfish sperm and chicken erythrocytes. The combination of low-temperature embedding to minimize sample damage [10], a DNA-specific EM stain to improve contrast [11,12], and high-resolution EM tomography using direct digital data acquisition has allowed chromatin structure to be examined in unprecedented detail [13•].

The use of such state-of-the-art approaches showed that 30 nm chromatin fibers follow highly contorted paths within the nucleus, and in areas of close packing, they appear to interdigitate. Detailed analyses of distinct fibers indicated that each fiber is a dinucleosomal ribbon that twists, turns, loops and folds in a continuously variable manner. Features seen in two-dimensional projections, such as cross-striations (previously interpreted as the projection of a helical coil), and putative 'end-on' views with 8–12 nucleosomes in peripheral radial arrangements, were found to be short-range local variations in the path of the ribbon when examined in their three-dimensional context.

In many cases, it was possible to discriminate between linker and nucleosome-associated DNA [13•]. Linker DNA connected nucleosomes across the fiber axis, rather than along it. In favorable reconstructions containing sequential nucleosomes, a 'zig-zag' path was often discerned. The separation between nucleosomes both parallel and perpendicular to the local fiber axis remains constant, with the fibers occupying, on average, a cylindrical space. However, as the nucleosomal ribbon folds in a highly localized manner, there does not appear to be a single 30 nm fiber structure. Rather, there is a general organization that appears quite flexible and could therefore assume the various structural states that must accompany the processes of DNA replication, transcription and chromosome condensation.

An understanding of these observations requires the consideration of the intrinsic heterogeneity in linker length.

Abbreviations

EM—electron microscope; **topo**—topoisomerase.

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Such variation affects the relative rotation of consecutive nucleosomes [14•]. Simulation of this variation produced structures strikingly similar to the chromatin fibers visualized in the tomographic reconstructions (CL Woodcock, SA Grigoryev, RA Horowitz, N Whitaker, unpublished data). It is important to realize that even subtle modifications of histones or non-nucleosomal proteins could easily affect linker packing and hence modulate the local structure of the 30 nm fiber.

How the 30 nm fiber folds into higher order condensed structures such as heterochromatin and linear chromosomes remains to be determined. This task has proven a formidable one: the higher order structures are extremely sensitive to preparation-induced artifacts and are extraordinarily complex. Currently, there are a number of models for higher order chromatin organization but relatively little definitive data. A similar approach to that used for the 30 nm fiber, making use of optimized sample preparation, staining, data collection and high-resolution EM tomographic imaging, is being used in our laboratory to produce a model of condensed chromosome structure in its native state.

Chromosome structural proteins

The *Polycomb* group

Higher order chromatin structure is mediated by the formation of protein and nucleic acid complexes. Genetic analysis has suggested that the *Drosophila Polycomb* gene family promotes the formation of higher order structure by encoding multimeric complexes [15]. These proteins are necessary for the repression of homeotic genes in tissues where expression would lead to inappropriate homeotic transformation. Recently, two of these proteins, Polycomb (Pc) and polyhomeotic, were shown to co-immunoprecipitate from solubilized ammonium sulfate pellets of nuclear extracts [16••]. Interestingly, 10–15 other proteins could also be immunoprecipitated with anti-Pc antibodies in a complex estimated by gel filtration to be 2000–5000 kDa. The proposal that Pc and polyhomeotic form a complex *in vivo* was strengthened by immunofluorescence results showing that the two proteins co-localized precisely on *Drosophila* salivary gland polytene chromosomes. While Pc can be localized to the regulatory sequences of homeotic genes, it does not appear to bind these sequences directly *in vitro* [17]. Therefore, these proteins may regulate the local higher order structure around homeotic genes by forming a complex, possibly with a number of other proteins.

Topoisomerase II

Perhaps the best studied non-histone chromosomal protein involved in higher order structure is DNA topoisomerase (topo) II, a 170 or 180 kDa enzyme that catalyzes a reaction in which one DNA helix passes through another. The localization of the 170 kDa form of topo II to the chromosome scaffold and the identification of DNA sequences that specifically bind to the scaffold *in vitro* (scaffold or matrix attachment regions) suggested that the enzyme might serve to organize the genome into loops bound to the scaffold at their ends by topo II [18]. To date, the scaffold remains a biochemical entity and its relevance to *in vivo* chromosome structure remains uncertain.

A number of recent reports have mapped the binding sites of topo II at transcribed and non-transcribed loci *in vivo*. Topo II binding sites can be mapped using the drug VM-26, which stabilizes the double-strand breaks generated by the enzyme; the positions of these breaks are then identified using indirect end-labeling of the DNA. *In vitro*, topo II is specifically localized to an A/T-rich consensus sequence; *in vivo*, however, it maps to internucleosomal linker DNA or nuclease-accessible 'open' chromatin [19,20•–22•]. When isolated nuclei, rather than whole cells, were treated with VM-26, the pattern and relative intensities of topo II sites changed dramatically; therefore results based on the use of isolated or extracted nuclei should be interpreted with caution. So far, *in vivo* topo II sites appear to fall into two categories: those that are not affected by transcription and those that disappear, or become much less frequently used, upon transcriptional activation. The latter class includes sites in the non-transcribed regions of the *Drosophila* actin, histone and *hsp70* genes, as well as sites within the transcribed regions of actin and *hsp70* [20•–22•]. The role these sites might have in transcription is not yet known. All of these experiments suggest that the localization of topo II in the interphase nucleus is highly regulated, with at least one feature of regulation being the position of nucleosomes. However, it is not at all clear why topo II concentrates at some specific linker DNA sites and not others. Sequencing of *in vivo* topo II sites at the histone locus suggested some preferred nucleotide positions in an 18 bp sequence, but no real consensus sequence [22•]. Given the size of the enzyme, it may be that only longer lengths of linker DNA are accessible to the enzyme [20•]. Alternatively, other factors might help guide the enzyme to bind at specific sites. In any case, the presence of multiple binding sites in each of the loci studied suggests that the proposed stoichiometry of one topo II molecule per chromatin loop attachment might underestimate the complexity of the structural organization of transcriptional regulation [23].

Topo II is required for the complete condensation of chromosomes during prophase *in vivo* and *in vitro* [24,25,26•]. The nature of this requirement was recently explored using a number of *in vitro* systems. Nuclei with low or undetectable levels of topo II (either chicken erythrocyte or *Xenopus* sperm nuclei) were added to mitotic extracts derived from colchicine-arrested chicken tissue culture cells or *Xenopus* egg extracts to test the role of topo II in chromosome assembly [27,28,29••]. Immunodepletion of topo II prevented condensation of the nuclei or naked DNA added to these extracts. However, substantial differences exist between the results obtained in the different studies. Chicken erythrocyte nuclei added to topo II depleted *Xenopus* extracts formed partially resolved, semi-condensed structures called 'precondensation chromosomes' [28]. *Xenopus* sperm nu-

clei added to topo II depleted *Xenopus* extracts formed only uncondensed chromatin [29••]. Erythrocyte nuclei added to topo II depleted mitotic chicken cell extracts did not form precondensation chromosomes either, but did compact to approximately half their original volume [27]. Exogenous topo II complemented the depleted *Xenopus* extracts, but not the depleted mitotic chicken cell extract. Complementation in the *Xenopus* extract required stoichiometric replacement of endogenous topo II levels, suggesting a structural role for the enzyme in condensation [28]. By contrast, extraction of condensed chromosomes under a variety of conditions that preserve the gross morphology of the chromosome removed all detectable topo II from the chromosome, suggesting that topo II is not required for the structural maintenance of the condensed state [29••]. The reconciliation of these different results is not yet possible; some of the disparity may be due to the different extracts and nuclear substrates used. It is also possible that multiple forms of topo II exist, not all of which function as chromosome structural proteins.

Evidence for this last hypothesis has come from direct *in vivo* localization of topo II during the cell cycle of the early *Drosophila* embryo [30••]. Enzymatically active rhodamine–topo II was injected into *Drosophila* embryos and its three-dimensional spatial localization was followed as a function of time. The enzyme was found to be generally chromosome-associated; however, separate populations of enzyme dissociated from the chromosomes and diffused into the cytoplasm after chromosome condensation and segregation. Overall, 70% of the enzyme present in the early prophase chromosome was lost by the end of mitosis. Therefore, most of the chromosomal enzyme appears to be partitioned into separate populations whose localization correlates with two known functions of topo II, but is not involved in maintaining the structural organization of the chromosome.

What is the molecular basis for this complexity? Some of the diversity may be explained by the presence of a second gene for topo II encoding a protein of 180 kDa with different biochemical properties from the 170 kDa enzyme [29••,31,32]. Immunofluorescence with monoclonal antibodies has localized the 180 kDa enzyme to the nucleoli of cultured cells, suggesting that this form might have a specific function in the transcription of rDNA [33]. This separation of activities is reminiscent of the situation in at least one prokaryote, where the function of type II topoisomerases in replication and segregation may be partitioned into two different enzymes, DNA gyrase and topo IV, respectively [34•].

However, much of the diversity in function of topo II may be explained by the striking number of post-translational modifications that occur in the enzyme. The observation of increased phosphorylation of topo II in mitotic cells suggested that mitotic kinases might regulate the activity of topo II *in vivo* [35]. The recent direct measurement of topo II activity in a *Xenopus in vitro* chromosome segregation system found that bulk topo II activity was highest in metaphase and decreased somewhat during anaphase [36••]. However, no evidence was found for a large change in activity during mitosis, but this experiment could not rule out the possibility that a subpopulation of chromosomal topo II was specifically activated at mitosis. Such complexity is suggested by the recent mapping of phosphorylation sites in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* topo II [37••,38••]. The modifications occur exclusively in the amino- and carboxyl-terminal ends of the enzyme and were detected only on serine in *S. pombe*, and on serine and threonine in *S. cerevisiae*. Most of the modified sites contain consensus sequences for phosphorylation by casein kinase II and no phosphorylation of *S. cerevisiae* topo II was detected in a casein kinase II mutant strain, consistent with previous results showing casein kinase II phosphorylation of topo II in *Drosophila* [39]. The role of phosphorylation in regulating the activity of topo II is not yet clear, as *in vitro*, phosphorylation is required for the mouse enzyme, only causes a threefold increase in activity of the *Drosophila* enzyme, and has no effect on the *S. pombe* enzyme [38••,39,40].

Conclusion

The last year has provided important insights into the structure of the 30 nm chromatin fiber and a few of the 100 or so non-histone chromosomal proteins. The theme that runs through many of the recent results is the variability and complexity of both the structure and its molecular components. The 'holy grail' of this field is the unification of structural observations with molecular function.

Note added in proof

The paper referred to in the text as CL Woodcock, SA Grigoryev, RA Horowitz, N Whitaker, unpublished data, has now been accepted for publication [41].

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Three-dimensional tomographic reconstructions of negatively stained isolated chromatin fibers show little evidence for large organized helical arrays of nucleosomes in the fiber, even where examination of stereo pair images suggested a helical arrangement. Significant flattening of the chromatin fiber occurs on the sample grid, suggesting at least one source of structural deformation in isolated fibers.

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This paper provides a preliminary report of three-dimensional tomographic reconstructions of preserved starfish sperm and chicken erythrocyte 30 nm chromatin fibers *in situ.* The fibers were stained with osmium ammine B, an electron-dense, nucleic acid specific stain, so the path of the DNA can be followed with certainty. The dominant folding pattern of nucleosomes appears to be a 'zig-zag', with the fiber filling, on average, a cylindrical volume. Nucleosomes appear to be connected by the linker DNA across the fiber axis.

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The complexity and dynamics of topo II binding sites *in vivo* are demonstrated at the *Drosophila hsp70*, histone and tRNA loci. Topo II binding sites coincide with micrococcal nuclease or DNase I hypersensitivity sites and many change dramatically upon trancriptional activation. In a series of careful control experiments, drug-stimulated cleavages detected by indirect end-labeling are shown to be specific and topo II dependent. When compared to *hsp70*, very little cleavage is observed throughout the histone locus, even in the histone scaffold attachment region.

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A thorough examination of the phosphorylation of topo II in *S. cerevisiae* suggests that the enzyme is phosphorylated on serine, and to a lesser extent threonine, residues, probably by casein kinase II. The enzyme shows a 6-fold to 10-fold increase in phosphorylation in mitosis over G_1, mostly due to an overall increase in phosphorylation, although some of the modifications appear to be either G_1 or M phase specific. The sites of phosphorylation are concentrated at the carboxyl-terminal end of the molecule, suggesting that this domain serves some regulatory function, but the effect of phosphorylation of enzyme activity in *S. cerevisiae* is not yet known.

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