A Two-Step Scaffolding Model for Mitotic Chromosome Assembly

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Summary

Topoisomerase IIα (topoIIα) and 13S condensin are both required for mitotic chromosome assembly. Here we show that they constitute the two main components of the chromosomal scaffold on histone-depleted chromosomes. The structural stability and chromosomal shape of the scaffolding toward harsh extraction procedures are shown to be mediated by ATP or its non-hydrolyzable analogs, but not ADP. TopoIIα and 13S condensin components immunolocalize to a radially restricted, longitudinal scaffolding in native-like chromosomes. Double staining for topoIIα and condensin generates a barber pole appearance of the scaffolding, where topoIIα- and condensin-enriched “beads” alternate; this structure appears to be generated by two juxtaposed, or coiled, chains. Cell cycle studies establish that 13S condensin appears not to be involved in the assembly of prophase chromatids; they lack this complex but contain a topoIIα-defined scaffold. Condensin associates only during the pro-to metaphase transition. This two-step assembly process is proposed to generate the barber pole appearance of the native-like scaffolding.

Introduction

Chromosomes appear constantly on the move and have a remarkable chameleonic behavior. Their structure is continuously adapted, locally and long range, to present genes for regulation and expression, to maintain and duplicate the genetic material with high fidelity, and to package and deliver it ever so carefully to both daughter cells. The plasticity of chromosome structure is also noted at mitosis, where the extent of condensation appears to depend on the length of the mitotic phase. Hence, chromosomal arms of rapidly dividing embryonic mitotic cells are longer and spindlier than those of somatic cells (Micheli et al., 1993), and, conversely, chromosomes super condense if mitosis is pharmacologically prolonged with inhibitors (Rieder and Palazzo, 1992). The process of chromosome condensation may not yield a unique structure, determined by a finite number of repeating interactions, as exemplified by viruses. This is perhaps best suggested by the finding that sister chromatids, certainly those of super-condensed chromosomes, are structurally related to each other by mirror symmetry (Baumgartner et al., 1991; Boy de la Tour and Laemmli, 1988). It follows that, if mitotic chromosomes were built by unique molecular interactions, then some would have to be of a nonnatural, opposite handedness in the paired sisters; chromosomes may include quasi-equivalent, flexible shape-determining interactions. What then is the process at mitosis that carves out chromosomal bodies from the dispersed chromatin mass of the interphase nucleus?

Chromosome structure appears to arise from a series of shape-determining crossties, termed scaffolding interactions (Laemmli et al., 1978). One early observation that inspired this notion is that chromosomes can enormously swell in vitro, under conditions that generally disperse and solubilize chromatin, but reversibly regain their normal morphology if physiological buffers are reapplied (Cole, 1967).

Attempts to identify these interactions led to the scaffolding-loop model. It was initially based on observations that gentle histone-depleted chromosomes maintain a large butterfly-shaped structure of a defined sedimentation behavior and consists of looped DNA held together by a network of nonhistone proteins, termed scaffolding (Adolph et al., 1977a, 1977b; Paulson and Laemmli, 1977). This model made a number of predictions for which evidence was later obtained: the existence of chromatin loops in unextracted chromosomes (Earnshaw and Laemmli, 1983; Marsden and Laemmli, 1979), the identification of a subset of scaffolding proteins (Lewis and Laemmli, 1982), and the discovery of DNA scaffold attachment regions (termed SARs) that may define the bases of the chromatin loops (Mirkovitch et al., 1984). While a definitive role for SARs in chromosome architecture is not established, results of inhibitor and structural studies provide good evidence for this notion (Strick and Laemmli, 1995; Saitoh and Laemmli, 1994; Strick et al., 2001; Hart and Laemmli, 1998).

The discreet subset of scaffolding proteins consists mainly of two major bands, termed Sc1 and Sc2 (Lewis and Laemmli, 1982). Sc1 was later identified as topoisomerase II (Earnshaw et al., 1985; Gasser et al., 1986), and further studies have implicated this protein in chromosome condensation (Adachi et al., 1991; Uemura et al., 1987). Topoisomerase II, which binds SARs specifically in vitro (Adachi et al., 1989), can relax positive and negative supercoils and catenate/decatenate tangled DNA (for review, see Wang, 1996). Sc2 was identified as a member of the SMC protein family (Saitoh et al., 1994; see below).

Remarkable progress in the field of in vitro chromosome assembly has led to the identification and characterization of the 13S condensin complex (Hirano et al., 1997; Hirano and Mitchison, 1994). This complex is composed of five different subunits, Smc4 (XCAP-C) and Smc2 (XCAP-E), which form a heterodimer, and three additional subunits, called XCAP-D2 (Eg7), XCAP-G, and XCAP-H (Hirano et al., 1997). The SMC proteins were...
originally identified in the budding yeast as gene products involved in the “structural maintenance of chromosomes” (Strunnikov et al., 1995) and are well conserved from bacteria to mammals (reviewed by Cobbe and Heck, 2000; Hirano, 2002). XCAP-H is a Xenopus ortholog of the Drosophila Barren protein, which was shown to be required for chromosome segregation (Bhat et al., 1996).

Interesting clues regarding mechanisms of chromosome condensation stem from the finding that 13S condensin can induce positive supercoiling of a DNA template (Kimura and Hirano, 1997). The positive supercoils arise from the introduction of a global positive writhe, rather than from a local twisting of DNA (Kimura et al., 1999). One of the models linking this global writhe of DNA to chromosome condensation suggests that 13S condensin, bound at intervals, tethers the DNA into topologically constrained loops, whose bases cooperatively interact to form a right-handed, solenoidal framework. This macromolecular folding is proposed to introduce the observed global DNA writhe. This model shares similarities with the loop-scaffolding model of chromosomes. Indeed, a relationship between the 13S condensin and the scaffolding is suggested by the observation that the Sc2 component of the scaffolding is a member of the SMC family (Saitoh et al., 1994).

In this study, we examined the relationship between the 13S condensin complex and the scaffolding by performing a detailed biochemical and structural analysis. The observations reported here allow for the formulation of a much more elaborate structural description of chromosomes.

Results

The Chromosomal Scaffolding Contains All 13S Condensin Components and Topoisomerase II

Scaffolding were isolated from purified chromosomes as described (Lewis and Laemmli, 1982), to study the relationship between this isolate and 13S condensin (Hirano et al., 1997). Toward this aim, chromosomes were isolated with polyamine-EDTA buffer, stabilized with CuSO4, and then extracted with 2 mg/ml dextran sulfate. Isolated scaffolding were run on a 7.5%–15% SDS-polyacrylamide gel and blotted to nitrocellulose membranes by the diffusion method (Bowen et al., 1980). Lanes 1 and 3, protein pattern of scaffoldings visualized by colloidal gold staining (gold). The main protein bands, Sc1 and Sc2, are indicated. Lane 2, immunoblot of hCAP-C, hCAP-E, and hBarren. Lane 4, immunoblot of topoII. Lane 5, immunoblot of hCAP-G and hEg7. (B) Stabilization of the scaffoldings with ATP and nonhydrolyzable analogs. Purified chromosomes were digested and stabilized by different cofactors (indicated), and the resulting scaffoldings were fractionated on a 7.5%–15% SDS-polyacrylamide gel, blotted, and stained. Top filter, immunoblot for topoII. Middle filter, immunoblot for hCAP-C, hCAP-E, and hBarren. Bottom filter, colloidal gold stain of the filter to verify the efficient histone depletion. Lanes 1 and 2, pattern of purified chromosomes with and without nuclease digestion, respectively. Lanes 3–8, pattern of scaffoldings isolated from digested chromosomes stabilized by different cofactors and extracted with 2 M NaCl. Cofactors used are as follows (cofactor, final concentration): lane 3 (CuSO4, 0.5 mM), lane 4 (AMP-PNP, 1 mM), lane 5 (ATP, 1 mM), lane 6 (ATP·S, 1 mM), lane 7 (ADP, 1 mM), and lane 8 (no cofactors). The scaffoldings in lanes 3–9 were obtained from the same amount of chromosomes as was loaded in lanes 1 and 2.
These results establish that the scaffolding is predominantly composed of topolα, the five condensin subunits, and, possibly, some unknown minor bands (contaminants?).

**The Stability of the Scaffolding Is Mediated by ATP or Its Analogs, but Not by ADP**

Scaffolding derived from chromosomes isolated in polyamine-EDTA buffer, unlike those isolated with Mg2+ or Ca2+ (Lewis and Laemmli, 1982). Although one may question a physiological role for Cu2+ ions in chromosome architecture (e.g., Hirano and Mitchison, 1993; Lewis and Laemmli, 1982), we considered it reasonable to suggest that Cu2+ (or Ca2+) may preserve preexisting interactions, acting as a short-distance crosslinker, rather than induce an artifact. Of note is a recent report that assigns an important function to Ca2+ ions in chromosome structure (Strick et al., 2001). We revisited this controversy and observed that stable scaffolding can be obtained under more-physiological conditions by exposure of polyamine-EDTA chromosomes to ATP or to its nonhydrolyzable analogs (ATPγS and AMP-PNP), but not to ADP. Figure 1B shows immunoblots of intact chromosomes (lanes 1 and 2) and scaffolding (lanes 3–8) exposed to different cofactors (indicated). The top filter of Figure 1B detects topolα, and the middle filter reveals hCAP-C, hCAP-E, and hBarren.

Examination of these blots first confirms our previous observation, namely, that polyamine-EDTA chromosomes are unstable (do not sediment) upon extraction with 2 M NaCl (lane 8) unless they have been exposed to Cu2+ ions (lane 3). The other lanes of these panels reveal that the scaffolding also resists extraction if chromosomes have been exposed to 1 mM of ATP (lane 5) or to the nonhydrolyzable analogs AMP-PNP (lane 4) or ATPγS (lane 6). In contrast, this substructure remains unstable (does not sediment) if ADP is used (lane 7).

Semi-quantitative comparison of these blots is possible, since the scaffolding protein fractions shown in lanes 3–8 were derived from the same amount of intact chromosomes loaded in lanes 1 and 2. Such a comparison of immunosignals establishes that the condensin components hCAP-C, hCAP-E, and hBarren partition quite quantitatively into the scaffolding, particularly following stabilization with the ATP analogs. The retention of topolα in the fraction is less complete and more variable, the highest retention being observed with ATPγS and Cu2+ ions. This is in line with our previous observation, where we estimated that about 60% of topolα partitions into the scaffolding, using Cu2+ stabilized chromosomes (Gasser et al., 1986). The scaffolding fractions shown in Figure 1 were obtained by extraction with 2 M NaCl. Similar results were obtained with the dextran sulfate extraction procedure at low ionic strength (data not shown).

The above results establish that the scaffolding proteins of isolated chromosomes exist in two different conformations governed by the presence or absence of ATP or its nonhydrolyzable analogs; this is experimentally reflected by two different stabilities toward biochemical extraction. That physiological cofactors such as ATP can stabilize the scaffolding suggests that this structure may reflect genuine aspects of the in vivo situation.

**Localization of the Scaffolding in Native-like Chromosomes**

The observation that condensin and topolα cosediment in extracted scaffolding begs the question of their structural relationship in native-like chromosomes. Immunofluorescence studies presented in Figure 2 show that hBarren (red) is sharply restricted within the blue (DAPI) chromosomal body. The hBarren signal highlights a central axis that passes through the centromere and extends longitudinally along the entire chromosomal length. Three different morphological classes of chromosomes were observed. The majority of the chromosomes have a beaded/coiled-like hBarren signal and their chromatids are closely juxtaposed (Figure 2A). Closely paired chromatids represent the “normal” morphology of chromosomes observed in unblocked mitotic cells. A few of the isolated chromosomes show X-shaped structures, if prepared from colcemid-blocked mitotic cells (Figure 2B). The hBarren signal of these split chromosomes is similar, although it often appears more extended, and has a less beaded/coiled-like configuration. Note that the term beaded/coiled-like (beaded only, hereafter) is used, since this pattern could arise from genuine coiling of a chain or from a thickening of a chain (see Discussion).

A few chromosomes super condense during the prolonged colcemid arrest (Figure 2C). Such structures are shorter and wider, and their hBarren signal is clearly coiled with opposite handedness in sister chromatids. This is best seen in Figure 2D, which shows a shaded, volume rendering of the particle of Figure 2C. This appearance is similar to that previously reported for super-condensed chromosomes immunostained for topolα (Boy de la Tour and Laemmli, 1988).

The subchromosomal signal of hBarren also applies to other condensin components. Figure 2E shows that the staining patterns for hEg7 are similar to those of hBarren (Figure 2A or 2B). This suggests that the observed restricted axial location of the immunosignal reflects that of the 13S condensin complex in isolated chromosomes.

Previous immunolocalization studies demonstrated that topolα is restricted to an axial-longitudinal, subchromosomal structure in isolated, partially expanded chromosomes (Earnshaw and Heck, 1985; Gasser et al., 1986). Figure 2F shows that, like condensin components, topolα of isolated native-like chromosomes is also longitudinally beaded and axially restricted. Hence, topolα and condensin are not only “biochemically linked” in scaffolding but also share a similar structural localization to a central axis in chromosomes.

A recent study, with cell lines that stably express a GFP-tagged topolα gene, observed that this protein quite homogeneously (not axially) associated with chromosomes (Christensen et al., 2002). Using these cell lines, we have confirmed their observation. However,
we have obtained strong evidence that this general binding is a consequence of an overabundance of topoII in these cells (see Supplemental Figure S2 at http://www.developmentalcell.com/cgi/content/full/4/4/467/DC1).

Localization of 13S Condensin in Mitotic Cells: End-on Views

The immunofluorescence studies described above were carried out with chromosomes isolated from colcemid-blocked HeLa cells that were spun onto a coverslip and then immunostained. Morphological studies with isolated chromosomes are advantageous; such preparations are physically thin (about 2–3 μm), allowing optimal optical resolution and also permitting efficient immunostaining and washing conditions. However, use of purified chromosomes raises concerns about isolation-dependent artifacts, such as rearrangements and extraction of proteins. Additionally, we noted that the chromosomal surface is deformed, presumably by adherence to the glass surfaces (data not shown). Is the restricted location of condensin and topoII an artifact arising from the isolation procedure of chromosomes or a peripheral deformation of bulk chromatin?

These concerns were addressed by immunostaining exponentially growing, unblocked mitotic cells. Figures 2G–2L show examples of stained mitotic cells. Although fluorescence micrographs of chromosomes in cells appear “fuzzier” than those of isolated particles, they clearly reveal an axially restricted, longitudinally beaded hBarren signal (Figures 2G and 2H; see also animation in Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/4/4/467/DC1). We noticed in the staining studies with fixed cells that a fraction of hBarren is not chromosome-bound, but cytoplasmic, and can be seen as a punctate signal (see Figure 2G). This “soluble” hBarren fraction is lost without alteration of the chromosomal pattern if cells are lysed by detergent prior to fixation (Figure 2; see also Figure 3). This panel displays a detailed rendering of a chromosome in a cell blocked with colcemid that often induces an extra level of coiling. Mitotic cells were also stained for topoII, confirming
that this protein also has a similar subchromosomal localization under those conditions (Figure 2J). Note that topol\(\alpha\) is generally enriched at centromeres, as previously observed (Warburton and Earnshaw, 1997).

Stained mitotic cells provide a unique opportunity to examine chromosomes end on if they are longitudinally oriented along the focusing/observation axis. Such end-on views display the chromosomal cross-section at the higher resolution of the \(x\)-\(y\) plane (about 0.3 \(\mu\)m). Most importantly, chromosomes viewed end on in cells are “freestanding” and are not deformed by adherence to the glass support. These end-on views clearly reveal the blue DAPI-stained spherical chromosomal bodies and the central, red hBarren (Figure 2K) or topol\(\alpha\) (Figure 2L) signals. To underscore this statement quantitatively, we carried out intensity line scans through the chromosomes and the central, red hBarren (Figure 2K) or topol\(\alpha\) (Figure 2L) signals. These tracings inserted into Figures 2K and 2L display the normalized intensity (12 bit data) for the red immunofluorescence and blue chromatin signals. Note that these radial intensity tracings reach

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<table>
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<tr>
<th>Interphase</th>
<th>Prophase</th>
<th>Metaphase</th>
<th>Anaphase</th>
<th>Telophase</th>
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**Figure 3. Cell Cycle Studies: Topol\(\alpha\), but Not 13S Condensin, Associated with Prophase Chromosomes?**

Exponentially growing HeLa S3 cells were immunostained for topol\(\alpha\), hBarren, or hCAP-E (Sc2) and counterstained with DAPI, as indicated. Images containing inter-, pro-, meta-, ana-, and telophase structures are shown; they were assembled from low-power (40\(\times\)1000) micrographs to allow comparison of signal intensities. Note that 13S condensin signals are weak at inter-, pro-, and telophase, in contrast to those at metaphase and anaphase. The bar represent 5 \(\mu\)m.
background levels toward the chromosomal periphery, where the blue chromatin signal is still at its maximum value. This analysis establishes that topoIIα and hBarren are predominantly confined within a tube of a diameter of around 35% that of the chromatid width (about 280 nm and 800 nm, respectively). The micrographs presented in Figure 2 were obtained from cells fixed with paraformaldehyde; identical results were obtained by cold-methanol fixation.

These observations demonstrate that the 13S condensin signal (hBarren and Eg7) and topoIIα are longitudinally beaded and radially restricted in chromosomes of intact mitotic cells.

Cell Cycle: Prophase Chromatid Formation Precedes Association of Condensin

The 13S condensin exists in the cytoplasm through most of the interphase stage of the cell cycle and then associates with chromatin during the process of mitotic condensation. This association is regulated by posttranslational phosphorylation of condensin and, possibly, of the chromatin substrate (for review, see Hirano, 2002).

TopoIIα, in contrast to condensin, is chromatin-bound during the entire cell cycle, while its cellular levels are regulated at the transcriptional level. The level of chromatin-bound topoIIα, however, is strongly reduced in the G1 phase (and also in G0) and increases during the G2-M transition (for review, see Warburton and Earnshaw, 1997).

We determined the timing of chromatin association of condensin (hBarren, hCAP-E/Sc2, and Eg7) and of topoIIα in exponentially growing HeLa cells and compared this with the processes of chromosome formation by DAPI staining. Since the immunofluorescence and DNA signals are highest in mitotic cells, we recorded cells at low magnification to encompass different cell stages in a single optical field; this procedure permits a meaningful comparison of the fluorescence intensities. The top and fourth rows of Figure 3 show the DAPI patterns at interphase and during the prophase, metaphase, anaphase, and telophase stages of mitosis. The second and fifth rows confirm that topoIIα is chromatin bound and is similarly strong at all mitotic stages and in some interphase nuclei; one of the nuclei displayed, however, lacks a detectable topoIIα signal and is thought to be in G1 (compare the fourth and fifth interphase rows).

As expected, the hBarren signal is strong in metaphase and anaphase chromosomes and is weak in interphase nuclei. Interestingly, however, this hBarren signal is very low in prophase and telophase chromosomes. The weak association of the condensin components with prophases and telophase chromosomes is somewhat unexpected, since inspection of the DAPI images show that well-formed chromatids are either already present or remain intact, respectively, during these stages of mitosis (see below). Quantitative estimates show that the mean chromatin-bound hBarren intensity is about 20-fold lower at these latter stages than in mitotic chromosomes. To address a concern that hBarren may not be representative of the cell cycle behavior of condensin or that it is immunologically hidden in prophase (but not in metaphase), we examined other condensin components. These experiments established that h-CAP-E/Sc2 (Figure 3, sixth row) and hEg7 (data not shown) behave similarly to hBarren, with very weak signals in pro-, telo-, and interphase, but strong signals in metaphase. These studies suggest that early events of chromosome condensation that lead to the formation of prophase chromatids may occur with interphase (not mitotic) levels of chromatin-bound condensin. Indeed, recent genetic and RNAi interference experiments suggest that condensin may work at a later stage of chromosome compaction (Hagstrom et al., 2002; Steffensen et al., 2001).

Radially Restricted Localization of TopoIIα in Prophase Chromatids

TopoIIα is radially restricted on a beaded chain in mitotic chromatids (Figure 2). Is topoIIα also subchromosomal in prophase chromatids? The DAPI image of prophase nuclei looks grape-like (Figure 3); this appearance results from the optical sectioning of prophase chromatids that span such nuclei. This is more evident from DAPI images of a prophase nucleus recorded at high magnification, where the grapes reflect cross-sections of chromatids; they often line the nuclear periphery (Figures 4A, 4D, and 4E). The width of a prophase chromatid is about half (0.4 μm) that of a mitotic chromosome (0.8 μm). Figures 4D–4G show the topoIIα and DAPI signals in an optical section of this same nucleus. Comparison of these patterns establishes that topoIIα is structurally localized to an axis within the prophase chromatids. This is evident from the more confined appearance of the topoIIα signals than the DAPI signals and is perhaps best noted at the nuclear periphery (compare Figure 4F with 4G) and in the stereo presentation (Figure 4H; see also animation in Supplemental Figure S3 at http://www.developmentalcell.com/cgi/content/full/4/4/467/DC1). These panels reveal that the topoIIα signal consists of bright foci (often found as doublets) from which lower intensity tracks extend. These foci are proposed to represent the enriched interaction of topoIIα within centromeric heterochromatin; this feature is also evident, possibly to a lesser extent, in mitotic chromosomes (Figure 2). The double nature of the topoIIα pattern must reflect the paired sister chromatids. Note that the axial topoIIα signal often appears tethered to the nuclear periphery, which, in a favorable case, can be traced as a loop (Figure 4F, arrowhead). This loop and other views suggest that the topoIIα pattern is also on a beaded chain at prophase. The hBarren pattern was also recorded for this prophase nucleus of Figure 4C.

As noted above, this latter pattern is weak in prophase and consists of a few dots that generally do not colocalize with chromosomes. These experiments establish important observations: topoIIα is subchromosomal, both in prophase and mitotic chromatids, and is enriched at centromeres. Additionally, prophase, as opposed to mitotic, chromosomes contain interphase levels of condensing, as judged by immunofluorescence. Are prophase chromatids assembled by a topoIIα-marked (defined?) structural axis that initiates from centromeres?

Barber Pole Appearance Mediated by TopoIIα and 13S Condensin

TopoIIα and 13S condensin are both components of the scaffolding (Figure 1), and both define a morphologically
A Two-Step Scaffolding Model

Figure 4. Prophase Chromosomes Have an Axis Containing TopoIIα, but Not 13S Condensin
(A–C) High-resolution micrographs of a prophase nucleus immunostained for topoIIα (B) and hBarren (C) and counterstained with DAPI (A), shown separately.
(D–G) Two optical sections are shown of a prophase nucleus stained as above (hBarren signal not shown). The top row (D and E) shows the DAPI patterns at two different depths of focus. The bottom row (F and G) displays the topoIIα patterns of (D) and (E), respectively. Note that the topoIIα signal is axial, in part beaded, and contains bright foci (doublets) that must represent the preferential enrichment of this protein at centromeres.
(H) A stereo pair of the topoIIα signal of the prophase nucleus shown in (A)–(C). Note that the topoIIα-defined axial elements are paired (sister chromatids) and are emanating from centromeric foci. The bars represent 5 μm.

similar axis in mitotic chromosomes (Figures 2 and 3), although only the topoIIα axis is seen in prophase chromatids. The structural relationship of topoIIα and condensin was studied by double immunofluorescence. Figure 5 shows isolated chromosomes stained for topoIIα (green) and hBarren or Eg7 (red); bulk chromatin is shown in blue (DAPI). Examination of these micrographs confirms that topoIIα and hBarren are both beaded and radially restricted but that their main signals are longitudinally somewhat out of phase. That is, the green, topoIIα-rich beads are generally followed by red beads enriched for hBarren (Figures 5A and 5B) or Eg7 (Figure 5C).

The chromosomes double stained for topoIIα and hBarren (Figures 5A and 5B) or Eg7 (Figure 5C) generate a barber pole-like appearance, which is indistinguishable in either combination.

Examination of either unmerged immunosignal confirms the observation that topoIIα and condensin define a continuous beaded/coil-like chain (Figures 5E and 5F; see also Figure 2), where each morphological bead may reflect either a thickening of the chain or a coil. Figures 5D and 5G show subregions of Figures 5A and 5B at higher magnification, while Figure 5H displays a side view, with the optical sections presented from top to
Figure 5. Barber Pole Appearance of Chromosomes Stained for TopoIIα and 13S Condensin

Isolated chromosomes (A–H) or cryosections (I and J) obtained from mitotic cells that were immunostained for topoIIα (all panels, green), hBarren ([A], [B], and [D]–[H], red), or Eg7 ([C], red) and counterstained with DAPI (blue). The antibody combinations that were used are indicated. (D) represents a region marked in [A]. (E) and (F) show the marked region (top) of (D) unmerged. (G) represents a region marked in (B). (H) displays a side projection along the focusing axis of the region marked in (B). The bars represent 1 μm.

(I) The top half shows a cryosection representing mainly crossviews of chromosomes that were stained for topoIIα (green), hBarren (red), and DAPI (blue). The bottom half of (I) is identical to the top half but lacks the DAPI signal. (J) is as (I) but provides longitudinal views of the chromosomes. The bars represent 5 μm.

bottom along the z-focusing axis. These panels clearly confirm the barber pole pattern.

We were concerned that the barber pole appearance might be an artifact caused by a mutually exclusive, competitive antibody behavior, where a bound antibody, e.g., against topoIIα, is locally preventing the subsequent binding of anti-hBarren antibodies. This possibility is unlikely, since the pattern observed was unaltered when the order and timing of the addition of the primary antibodies were varied (data not shown). Moreover, if antibody interference were to occur, then images obtained by double or single staining should differ; this was found not to be the case.

To ascertain that the red/green pattern was not caused by lateral chromatic aberration, we determined this parameter with the help of multicolored fluorescent beads. A chromatic shift in the x-y plane of maximally one pixel (pixel resolution, 0.045 μm) was measured for the green (528 nm FITC) and red (617 nm) channels. Since the peak to peak distance of the barber pole pattern is generally much larger (0.3–0.4 μm), we can neglect this optical imperfection.

The Barber Pole Pattern Is Observed in Cryosectioned Mitotic Cells

Is the barber pole pattern also observed in chromosomes of unblocked, mitotic cells? This question was addressed by preparing cryosections from fixed cells
that were subsequently immunostained (Robinson et al., 2001). This procedure, although rarely applied, provides a number of advantages: cryosections provide native-like slices through cells; they represent physical (not optical) sections. Hence, views of the chromosomal interior obtained by physical or optical sectioning can be compared. Cryosections are thin (about 200 nm), permitting improved resolution over those obtained by optical sectioning (600 nm) and, in addition, allowing optimal antibody penetration.

Figure 5 shows images of cryosectioned, double immunostained chromosomes prepared from fixed cells. Figures 5I and 5J show cross and longitudinal slices through chromosomes, respectively. The hBarren signal is red, the topoIIα signal is green, and bulk chromatin is blue. The cross-sections of Figure 5I confirm that hBarren and topoIIα are radially very restricted within the blue chromosomal tube. Closer inspection further demonstrates that hBarren and topoIIα are closely juxtaposed but that the centers of intensity of their signals are not overlapping. This is best observed in the bottom part of Figure 5I, where the DAPI signal was omitted. Note that the strength of the red and green signals varies in such crossviews because of the longitudinal barber pole pattern. This is expected, since cross-sections with a thickness of 200 nm do not necessarily encompass both a green and red bead, which are about 400 nm distant (see Supplemental Figure S5 at http://www.developmentalcell.com/cgi/content/full/4/4/467/DC1).

Figure 5J shows a cryosection that generated a longitudinal, physical slice through many chromosomal arms and the centromeres. As noted above, centromeres are generally enriched for topoIIα, yielding a strong green signal, which can be observed in cryosections as well. This longitudinal cryosection also confirms the barber pole appearance. This is perhaps best noted in the bottom part of Figure 5J (no DAPI signal). The barber pole pattern of Figure 5J is less regular than that of intact chromosomes. As above, this is expected; cryosections are thin (about 200 nm), and, hence, a longitudinal slice does not necessarily include the entire barber pole axis.

We consider comparative morphological studies, where the preparation and analysis methods are varied, important. This approach allows an identification of those structural features that are robust and likely more genuine and of those that are more fastidious and, possibly, reflective of an artifact. The micrographs obtained by cryosectioning of mitotic cells confirm, by yet another technique and preparation method, that the structural features described here are very robust. These results establish that topoIIα and condensin are localized in mitotic chromosomes to radially restricted chains, which are composed of out of phase beads, where topoIIα or condensin is alternatively enriched.

The ATP Scaffolding Retains Detailed Structural Features and a Chromosomal Shape

The chromosomal scaffolds extracted from chromosomes digested with nuclease or from histone-depleted chromosomes retaining their DNA complement, remain structurally quite intact. That is, these scaffolds maintain not only a general chromosomal shape, but they also preserve a beaded substructure along their arms and a barber pole appearance. The structures displayed in Figure 6 were obtained by extraction with dextran sulfate; the immunosignal of topoIIα is green (except Figure 6D), and those of hBarren red and DNA are blue. Figure 6A displays the large blue DNA halos that result from histone depletion and the barber pole appearance of the central scaffolding. This latter structure expands longitudinally during extraction with dextran sulfate to yield a “discontinuous” appearance; a more compact/continuous appearance is obtained by extraction with 2 M NaCl (data not shown). Figures 6B–6F show DNA-depleted isolated scaffoldings (dextran sulfate extraction) that were immunostained singly or doubly for topoIIα and hBarren. The scaffolding preserves structural features of intact chromosomes remarkably well, such as the pairing of the sister chromatids at centromeres, the beaded/coiled pattern of the arms, and the barber pole appearance. The retention of these structural features inspires confidence that the scaffolding obtained from chromosomes structurally reflects the in vivo situation.

Discussion

TopoIIα and 13S condensin are abundant components of mitotic chromosomes required for their assembly from interphase nuclei. Both harbor ATP-dependent catalytic activities that can alter, albeit in different ways, DNA topology. TopoIIα removes positive or negative DNA supercoils and can catenate or decatenate DNA strands (Wang, 1996). Conversely, 13S condensin introduces positive supercoils (Hirano, 2002). How then do these topological DNA handlers exert a shape-determining function that carves out the shaped mitotic chromosomes, literally, colored bodies, from the homogenous mass of the interphase? Do these enzymatic activities not only encompass a shape-determining role to compact the chromatin fiber, but also apply a shape-maintaining/structural function in the assembled chromosomes? Toward an elucidation of these questions, we immunolocalized topoIIα and 13S condensin in mitotic chromosomes, followed these proteins around the cell cycle, and reexamined biochemically the relationship of topoIIα and 13S condensin with the chromosomal scaffolding. The data presented here establish that the scaffolding-loop model applies to near-native chromosomes and suggest further that 13S condensin appears not involved in prophase chromosome formation, as judged by immunofluorescence.

The Barber Pole Subchromosomal, Axial Location of TopoIIα and 13S Condensin

For immunolocalizations mitotic chromosomes were either harshly dephased of histones or maintained in a near-native state. Despite this range of mitotic material and the use of widely different preparation techniques, the micrographs obtained congruently establish that topoIIα and 13S condensin are localized to an axially
Figure 6. The Barber Pole Is Preserved in Histone-Depleted Chromosomes and Scaffoldings

(A) Isolated chromosomes stabilized by ATP-γS were extracted with dextran sulfate (2 mg/ml), spun onto a coverslip, fixed, stained for hBarren (red) and topoII (green), and counterstained for DAPI (blue).

(B–F) Scaffoldings were isolated from nuclease-digested chromosomes with dextran sulfate, spun onto a coverslip, fixed, and stained for hBarren ([B] and [C], red) or TopoII ([D], red) or double stained (E and F) for hBarren (red) and topoII (green). Although these samples (B–F) were counterstained for DAPI (blue), signals were at background level. Note the preservation of the chromosomal shape and of the beaded and barber pole appearance. The bars represent 1 μm.

restricted, longitudinally beaded chain that extends from telomere to telomere through the chromosomal body. The radially restricted location of these proteins is perhaps best evidenced by the end-on views of chromosomes in mitotic cells (Figure 2) and in cryosections (Figure 4). Immunostaining patterns of hCAP-E and hCAP-H (hBarren) extend those reported by Cabello et al. (2001) and Schmiesing et al. (2000). Moreover, while this manuscript was being edited, a study demonstrated that GFP-tagged topoII localized in the living cells to a subchromosomal region (Tavormina et al., 2002). Hence, near-native mitotic chromosomes contain a subchromosomal element defined by chromatin enriched for topoII and 13S condensin.

Double staining for topoII and 13S condensin components (hBarren, Eg7, or Sc2) generated a striking barber pole pattern. This appearance arises from two central chains that have thickened, topoII- or condensin-enriched regions (beads), which are longitudinally somewhat out of phase. The limits of optical resolution do not allow us to distinguish whether the red and green chains are coiled, as in a genuine barber pole or in more parallel juxtaposed chains (see Supplemental Figures 4S and 5S at http://www.developmentalcell.com/cgi/content/full/4/4/467/DC1).

Clear coiling, however, is observed in the hBarren-stained, super-condensed chromosomes, which arise during a prolonged mitotic block. In line with a previous study, where topoII was immunolocalized in partly swollen, super-condensed chromosomes (Boy de la Tour and Laemmli, 1988), we observed here that the hBarren signal is also coiled with opposite handedness in sister chromatids (Figure 2).

The barber pole may be a reflection of different preferences for DNA sequences or epigenetic chromatin of topoII and condensin. Previous studies demonstrated that topoII preferentially localizes to AT-rich DNA; this is manifested by its enrichment in Q bands (Saitoh and Laemmli, 1994). Does condensin associate with the more GC-rich R bands, that is, is the barber pole a Q/R-banding pattern?

The Relationship of the Scaffolding and 13S Condensin

TopoII and 13S condensin are quantitatively retained in an ATP-dependent way in isolated scaffoldings (as judged by immunoblotting), despite harsh extraction procedures. In the presence of such ATP nucleotides, topoII is known to form a closed clamp by capturing DNA in the central hole formed between its dimers (Roca and Wang, 1992). This clamp, which resists dissociation by high salt, could bring about the biochemical stability of the scaffolding. Of course, a conformational change of 13S condensin known to be induced by ATP (Hirano, 2002) could also be involved.

ATP-dependent interactions are generally considered to reflect biologically significant/native states. Given this notion and the strength of the presented structural studies, we consider it reasonable to propose that the biochemical scaffolding is reflective of a genuine network of interactions created or altered during the process of shape determination (scaffolding) that brings about chromosome condensation. This scaffolding may also have a shape-maintaining (scaffold), structural role in the final chromosomes. Experimentally it is difficult to
settle this point, since isolated chromosomes are structurally stabilized by general fiber-fiber interactions (chromatin is sticky). Hence, it is difficult to distinguish between the structural contributions made by both topolκ and 13S condensin (if any) from the general stickiness of the chromatin fiber; the distinction between shaping and maintaining chromosome morphology is not always appreciated in the literature and is a source of a gratuitous controversy.

**Prophase Chromosomes Contain a Topolκ-Defined Axis, but Lack 13S Condensin**

The immunosignal of prophase nuclei for 13S condensin is low and similar to that of interphase nuclei; it is, at both stages, composed of low-intensity granules (background) that generally are not correlated with DNA-dense regions (Figures 3 and 4).

Is 13S condensin lost from, or somehow “hidden” in, prophase nuclei? Although not definitively ruled out, such possibilities appear unlikely for the following reasons: the low 13S condensin signal at prophase is independent of cell type (HeLa and 293), antibodies (hBarren, Sc2, and Eg7), fixation protocols (formaldehyde and methanol), and whether fixed cells were prepared by whole-mount or by cryosectioning methods (data not shown).

In contrast to 13S condensin, topolκ is localized to a subchromosomal axis in prophase chromosomes (Figure 4). The topolκ signal consists of conspicuous bright doublets from where chain-like, often-beaded signals extend into each prophase chromatid. These bright doublets are proposed to represent the enriched association of topolκ with centromeric sequences. This feature is also observed with metaphase chromosomes, although centromeres appear less bright relative to the arms in this case. Examination of the DAPI signal shows that the body of prophase chromosomes is well defined and rod-like, of a width less than that of mitotic chromosomes (about 0.5 μm and 0.8 μm, respectively). Presumably, the thickening of the latter is a consequence of the shortening of the chromosomal length during the pro- to metaphase transition. Is 13S condensin only mediating this final condensation step?

Genetic studies are consistent with the view that 13S condensin acts late in mitosis. Studies with *Drosophila* flies defective for SMC4 (the ortholog of hCAP-C) showed that mitotic chromosomes are condensed quite normally in length but that sister resolution is disrupted (Steffensen et al., 2001). In addition, C. elegans embryos depleted for SMC4/MIX by RNAi display nearly normal, rod-shaped metaphase chromosomes but again fail to segregate sister chromatids at anaphase. We would like to emphasize that, although the major phenotype of condensin mutations is manifested in segregation (Hastrom et al., 2002), a main role for condensin in chromosome condensation is not ruled out. It is also possible that the low condensin level of prophase chromosomes is functionally relevant.

**Structural Role for Topolκ?**

If condensin acts during the pro- to metaphase transition, as suggested by the immunofluorescence data, how then are prophasic chromatids assembled? Does topolκ play a dual structural/enzymatic role in condensation? Recent photobleaching-recovery experiments of live cells demonstrate that GFP-tagged topolκ rapidly exchanges from centromeres and the chromosomal arms in mitosis (Christensen et al., 2002; Tavormina et al., 2002). The papers suggested that a mobile behavior is incompatible with a structural, scaffolding role for topolκ, and others have argued similarly, albeit on different experimental grounds (Hirano and Mitchison, 1993). We think that the plasticity of chromosomes may demand dynamically acting structural components, and, as pointed out above, a formal distinction between shape-determining and shape-maintaining roles is experimentally difficult. We consider the roles of topolκ (also condensin) as open.

How could topolκ or, for that matter, any other protein(s) bring about chromosome individualization? The following model is stimulated by the notion that topolκ becomes a heterochromatin, centromere-enriched protein starting in prophase, from where it extends as a subchromosomal chain into the arms (see Figure 4 and the animation in Supplemental Figure 3S; Tavormina et al., 2002). Hence, one may view the topolκ-marked (topolκ-determined?) chain of prophase chromosomes as an extension of centric chromatin, where the latter may be composed of interspersed, heterochromatic attachment regions (SARs?). We suggest that the heterochromatic state of these attachment regions is unstable/dynamic but that pairing and further assembly stabilize this interaction (heterochromatin is sticky). We propose that centromeric chromatin serves in mitosis as a seed (a sink rich of heterochromatinizing factors) that initiates this aggregation reaction (illustrated in Figure 7). It is reasonable to surmise that, energetically, the most stable state is obtained upon maximization of intra-, rather than inter-, chromosomal interactions. This process, therefore, leads first to chromosome/chromatid individualization, since there is only a single seed per chromatid (two per chromosome). Catenated/knotted chromatin strands counteract individualization; such linked strands, however, are under mechanical pull and should snap toward their respective aggregation center upon decatenation by topolκ (illustrated in Figure 7).

The one seed-per-chromatid model automatically untangles interchromosomal links. The directionality of catenation-decatenation of topolκ depends on DNA concentration and is shifted toward decatenation at lower concentration (Wang, 1996). The aggregation process increases DNA concentration in the intrachromosomal territory around the seed, while diminishing this value in the interchromosomal space. Thus, intrachromosomal catenates will be reduced as a consequence of condensation, while intrachromosomal (also inter-chromatid) catenates should be increased.

Why are rod-shaped chromatids, rather than spherical structures, created by this process? One possible rationale to obtain rod-shaped structures would be to propose that chromatin is strung between nuclear tethers (peripheral tethers are shown) that guide directional aggregation. Moreover, prophasic sister chromatids are still linked by cohesin bridges (Waizenegger et al., 2000), which must impose further mechanical constraints; sisters must condense in concert, and rod formation may be the consequence of this.
This illustration summarizes the data obtained and includes two highly speculative intermediates. DNA, blue; the position of topoIIα, green; the position of condensin, red.

(Pre-prophase?) A highly speculative model leading to chromatid individualization (sister chromatids not shown). The model proposes that the centric chromatin serves as a seed/sink that drives the assembly of interspersed, heterochromatic attachment fragment into the topoIIα-marked chain; it is unclear whether topoIIα plays a structural role.

(Prophase) The bright foci of the topoIIα signal at centromeres (CEN) and the topoIIα-marked chain are shown. This chain may be viewed as a mitotic extension of centric heterochromatin into the chromatid arms. This step of condensation yields prophase chromatids that are longer and thinner than those of metaphase. Sister chromatids are shown.

(Intermediate?) Condensin associates during the second step of condensation via an unknown intermediate, where 13S condensin may bind in batches to the surface/periphery of prophase chromatids. This is purely hypothetical.

(Metaphase) This last step results in the observed barber pole appearance of metaphase chromosomes. It is structurally unclear whether coiling of the topoIIα- or condensin-marked chains, stacking, or some other structural transformation generates the barber pole appearance.

**Making the Barber Pole Pattern**

The barber pole pattern observed in mitotic chromosomes stained for topoIIα and 13S condensin is likely to be the structural consequence of the two-step process of condensation proposed here (illustrated in Figure 7). The first step is the shaping of chromatids during prophase, which is accompanied by formation of the topoIIα axis. The second step is the shortening and thickening of the chromatids during the transition to metaphase, which is accompanied by the binding of 13S condensin and the appearance of the scaffolding barber pole. We do not know where 13S condensin initially binds, but Figure 7 depicts one possible hypothetical intermediate, where 13S condensin is bound to the periphery of the chromatid arms, possibly in a patchy fashion. Many other binding modes are possible; one attractive thought is to propose that 13S condensin replaces cohesin. In general terms, we propose that condensation during the pro- to metaphase transition brings chromatin beads enriched for 13S condensin together with those of topoIIα, as reflected by the barber pole appearance of the scaffolding. This could be achieved by winding the chains defined by topoIIα and 13S condensin, a process that could possibly be mediated by the positive supercoiling activity of 13S condensin. Alternatively, the topoIIα and 13S condensin beads could somehow stack, perhaps as a result of protein-protein interactions between these complexes. In line with this general model is a recent study, which suggests that DNA sequences at the periphery of prophase chromatids move to the center of metaphase chromatids, forming an axis-like structure during this condensation step (Dietzel and Belmont, 2001).

Finally, the data presented here provide strong evidence for the scaffolding-loop model in native chromosomes. We further demonstrate that chromosome condensation occurs in a two-step process, implicating first topoIIα and then 13S condensin. This two-step process, we propose, generates the barber pole structure of the stained scaffolding within native chromosomes. The enzymology of topoisomerase II is well studied (Wang, 1996), many molecular details of 13S condensin are known (Hirano, 2002), and we entertain a much better global picture of chromosome structure. The next challenge is to extend structural studies to a higher resolution and to understand how the molecular details of these activities are translated at mitosis into the grand finale: the making of chromosomal bodies.

**Experimental Procedures**

**Antibody Preparation**

Antibody preparation is described in the Supplemental Data at http://www.developmentalcell.com/cgi/content/full/4/4/467/DC1.

**Metaphase Chromosome Isolation and Chromosome Scaffolding Preparation**

Details are available in the Supplemental Data at http://www.developmentalcell.com/cgi/content/full/4/4/467/DC1.

**Immunofluorescence Staining of Isolated Chromosomes and HeLaS3 Cells**

For human Barron (hCAP-H) staining, HeLa S3 cells were grown on coverslips coated with polylysine (Serva). The cells were washed in PBS and fixed with freshly prepared 2% paraformaldehyde in XBE2 buffer (10 mM HEPES [pH 7.7], 2 mM MgCl2, 100 mM KCl, and 5 mM EGTA) for 15 min at room temperature. The fixed cells were then treated with 0.5 mg/ml NaBH4 (Fluka) in XBE2 for 5 min, rinsed with XBE2 (twice for 5 min), and permeabilized with 0.5% Triton X-100 (Biochem) in XBE2 buffer. After being washed twice for 5 min with XBE2, the cells were incubated with 3% normal goat serum (NGS; Nordic Immunology, The Netherlands) in XBE2 for 30 min at room temperature. The cells were incubated with 3000-fold-diluted anti-human Barron serum, 1% NGS, and 0.05% Tween 20 in XBE2 buffer at room temperature for 1 hr. After washing with XBE2 (five times for 3 min), the cells were incubated with 1000-fold-diluted...
anti-rabbit Cy3-tabeled goat IgG (Jackson Immunological Laboratories, West Germany) and 0.05% Tween 20 in XBE2 buffer at room temperature for 1 h. After extensive washing with XBE2 (six times for 3 min), the cells were mounted in PPD (10 mM HEPES [pH 7.7] 2 mM MgCl₂, 100 mM KCl, 5 mM EGTA, 78% glycerol, 1 mg/ml paraphenylene diamine [Sigma], and 1 μg/ml DAPI [Boehringer]) and the coverslips were sealed with a rapid epoxy glue (Araldit; Ciba, Switzerland). In some experiments, to remove free 135 condensin, we extracted HeLa cells with 0.1% Triton X-100 (Calbiochem) in XBE2 containing 0.1% Trasylol (Bayer), 0.5% Thiodiglycol, 100 nM Microcystin LR (Calbiochem), and 0.1 mM PMSF (Sigma) at 0°C for 5 min and washed them with the same buffer without Triton X-100 (twice for 3 min at 0°C).

For individual chromosome staining, isolated chromosomes were diluted in XBE2 and fixed by adding 1/10 volume of freshly prepared 8% paraformaldehyde (final, 0.8%) at room temperature for 15 min. The fixed chromosomes were spun onto polylysine-coated coverslips through XBE2 containing 30% sucrose and stained as described above. Similar staining patterns were obtained by methanol fixation (–20°C, 7 min) (Hirano and Mitchison, 1994), instead of paraformaldehyde fixation.

Cyrosectioning and Immunofluorescence Staining ofSemithin Section
HeLa cells were extracted in HKMC buffer (10 mM HEPES-KOH [pH 7.5], 100 mM KCl, 2 mM MgCl₂, 0.5 mM CaCl₂) containing 0.1% Triton X-100, 0.1% Trasylol, 0.5%MgCl₂, 100 nM Microcystin LR, and 0.1 mM PMSF at 0°C for 5 min and fixed with freshly prepared 4% paraformaldehyde in HKMC buffer (10 mM HEPES-KOH [pH 7.5], 100 mM KCl, 2 mM MgCl₂, and 0.5 mM CaCl₂) at room temperature for 15 min. The fixed cells were then treated with 0.5 mg/ml NaBH₄ (Fluka) in HKMC for 5 min, rinsed with HKMC (twice for 5 min), and permeabilized with 0.5% Triton X-100 in HKMC buffer. After being washed twice for 5 min with HKM buffer (10 mM HEPES-KOH [pH 7.5], 100 mM KCl, and 2 mM MgCl₂), the cells were stained with hBarren and topoisomerase II antibodies as primary antibodies and Alexa 488 G4R and Alexa 594 G4M antibodies (Molecular Probes) as secondary antibodies. After five washes with HKM buffer, the stained cells were suspended in HKM buffer containing 2.3 M sucrose. After penetration of sucrose into the cells, the cells were quickly frozen in liquid nitrogen and kept in it until sectioning. Sectioning was done with a diamond knife (Diatome, Switzerland) on a Leica EM FCS apparatus (Leica, Austria) at –90°C. The 200 nm semithin sections were collected and transferred to polylysine-coated coverslips with a drop of HKM buffer containing 2.3 M sucrose. The coverslips were mounted and sealed as described above. In some experiments, the fixed cells were frozen and cut before staining. In this case, the sections transferred to coverslips were stained as described above.

Microscopy and 3D Reconstruction
Image stacks were recorded with a DeltaVision-equipped (Applied Precision) Olympus X170 microscope, with a step size in the focusing axis of 0.1 μm. An Olympus 100×1.35 UPlanApo objective, which was combined with a 1.5× Optovar magnification lens, was used. The voxel dimension was 45 nm and 100 nm in the x-y and z directions, respectively.

Data stacks were deconvoluted with SoftWorks and an experimentally determined point spread function (Agard et al., 1989). The longitudinal chromatic aberration was corrected by shifting the different channels relative to each other. The measured chromatic shift was 0.4 μm between the blue and green channels and 0.5 μm between the blue and red channels. These values were obtained with the help of three-color beads of 0.1 μm diameter (Molecular Probes). The lateral chromatic aberration was measured to be negligible. All 3D reconstructions were performed with SoftWorks, with the exception of Figure 2D. The surface rendering shown was obtained with Imaris3 (Bitplane, Switzerland) with the Isosurface tool. It represents the three-dimensional surface of the hBarren signal above a certain threshold value.

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