A Postprophase Topoisomerase II–dependent Chromatid Core Separation Step in the Formation of Metaphase Chromosomes

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Abstract. Metaphase chromatids are believed to consist of loops of chromatin anchored to a central scaffold, of which a major component is the decatenatory enzyme DNA topoisomerase II. Silver impregnation selectively stains an axial element of metaphase and anaphase chromatids; but we find that in earlier stages of mitosis, silver staining reveals an initially single, folded midline structure, which separates at prometaphase to form two chromatid axes. Inhibition of topoisomerase II prevents this separation, and also prevents the contraction of chromatids that occurs when metaphase is arrested. Immunolocalization of topoisomerase IIα reveals chromatid cores analogous to those seen with silver staining. We conclude that the chromatid cores in early mitosis form a single structure, constrained by DNA catenations, which must separate before metaphase chromatids can be resolved.

ODELS of mammalian chromosome structure must account for the 10,000-fold packing of nuclear DNA at metaphase (Mazia, 1963; Comings and Riggs, 1971; Sedat and Manuelides, 1977). One factor involved, identified very early on, is the helical coiling of an axial chromatid element; the first century of work on this topic is reviewed by Manton (1950). Another factor is the structural subunit, revealed in micrographs of interface-spread, dehistonized chromosomes, of DNA loops radiating from a proteinaceous chromosome scaffold (Paulson and Laemmli, 1977; Mullinger and Johnson, 1979). The radial loop and helical coiling elements are unified in a current model of chromatin organization, in which DNA loops of ~50–100 kb are anchored to a network of nonhistone proteins, which in interphase forms a nuclear matrix and in mitosis condenses to form a chromosome scaffold, which itself folds in a helical pattern during the last stage of metaphase chromosome packing (Mullinger and Johnson, 1980; Rattner and Lin, 1985; Boy de la Tour and Laemmli, 1988), with different degrees of folding producing transversely banded structures on staining (Saitoh and Laemmli, 1994).

A major component of the chromosome scaffold is

DNA topoisomerase II, an enzyme capable of passing one DNA helix through another via a transient double-stranded break (Earnshaw and Heck, 1985; Earnshaw et al., 1985; Gasser and Laemmli, 1986). This is the only known eukaryote enzyme capable of decatenating double-stranded DNA; such decatenation is necessary to separate daughter strands of DNA, which inevitably become catenated in replication (for review see Cook, 1991). Yeast and *Drosophila* have only one form of topoisomerase II; mammalian cells have α and β isoforms, encoded by different genes, the former being a 170-kD protein most abundant in G_2 and mitosis, the latter being a 180-kD protein with no cycle dependence (Woessner et al., 1991). Topoisomerase II binds selectively to scaffold-attached regions of DNA (Mirkovitch et al., 1984; Laemmli et al., 1992). These are AT-rich regions with narrow minor grooves and bent conformation (Homberger, 1989); they appear to lie at the base of DNA loops protruding from the scaffold (Saitoh and Laemmli, 1994).

Full decatenation of these loops in sister chromatids, topologically constrained by their interactions with the scaffold, must occur before the start of anaphase. Minichromosomes in yeast often undergo complete decatenation quite soon after replication, well before anaphase begins (Koshland and Hartwell, 1987). However, normal full-size chromatids are connected till the end of metaphase by some catenations, which must be removed for segregation to proceed. This is indicated by the sensitivity of chromatid segregation to temperature-sensitive *Top2* mutations in yeast (di Nardo et al., 1984; Uemura et al., 1987). In higher eukaryotic cells, where no suitable topoisomerase II mutants exist, specific inhibitors of topoisomerase II—etoposide or ICRF-193—have analogous effects; mitotic segre-

gation is prevented in mammalian somatic cells (Downes et al., 1991; Clarke et al., 1993), in *Drosophila* embryos (Buchenau et al., 1993), and in cell-free systems based on *Xenopus* egg extracts (Shamu and Murray, 1992).

But, though some catenation of sister chromatids persists to metaphase, much catenation appears to be removed earlier in mitosis, since topoisomerase II activity is necessary for full mitotic condensation to occur. In fission yeast, temperature-sensitive *Top2* mutants are unable to complete chromosome condensation at the restrictive temperature, but enter a prophase-like state (Uemura et al., 1987). In *Xenopus* egg extracts, topoisomerase II inhibitors (Newport and Spann, 1987; Hirano and Mitchison, 1991) or immunodepletion of topoisomerase II (Wood and Earnshaw, 1990; Adachi et al., 1991) prevents complete condensation of chromosomes. In mammalian cells, matters are complicated by the existence of a temporary G_2 topoisomerase II–dependent checkpoint that regulates entry into mitosis; but when this checkpoint is evaded, cells that attempt mitotic condensation produce very elongated, imperfectly condensed chromosomal structures (Downes et al., 1994). So, the role of topoisomerase II in achieving full condensation is enzymatic, not structural. Likewise, extraction of topoisomerase II from compacted chromosomes does not affect morphology (Hirano and Mitchison, 1993).

Silver staining of chromosomes reveals chromosome cores that are sensitive to proteinase, but not DNase (Howell and Hsu, 1979); and isolated chromosome scaffolds have argentophilic properties (Earnshaw and Laemmli, 1984). Silver staining is an impregnation technique: as in photography, a redox reaction involving silver ions is autocatalytic, thus enlarging the areas that are stained without modifying their shape. We have analyzed the development and organization of these chromosome cores through the somatic cell cycle using Indian muntjac cells, which have conveniently large chromosomes; we have used silver staining and also immunolocalization of topoisomerase IIα. We have investigated the role of topoisomerase II in these processes, using the topoisomerase II inhibitor ICRF-193. This is one of a series of bisdioxopiperazines, which arrest topoisomerase II action after the conformational change induced by ATP binding but before it creates DNA strand breaks (Roca et al., 1994). The use of ICRF-193 allows decatenation to be inhibited with no collateral effects arising from DNA damage (Downes et al., 1994).

Materials and Methods

Cell Culture, Synchrony, and Fusion

SVM cells, an SV-40-transformed line of Indian muntjac (*Muntiacus muntjak*) fibroblasts (Pillidge et al., 1986), were maintained in monolayer cultures at 37°C in MEM supplemented with nonessential amino acids and 10% FCS. Small numbers of mitotic cells were obtained by brief arrest with 80 psi nitrous oxide; mitotic populations for cell fusion were obtained by thymidine arrest followed by automatic nitrous oxide synchrony (Downes et al., 1987).

For prematurely condensed chromosome (PCC)[1] experiments, mitotic

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1. *Abbreviations used in this paper*: PCC, prematurely condensed chromosome; SCC, sister chromatid core.

SVM cells were fused with random cultures of SVM according to the standard procedure of Johnson and Rao (1970). In some experiments, 2 μg/ml of ICRF-193 was present during the fusion with Sendai virus at 4°C and during the 30–90-min incubations at 37°C, which induced PCC.

Silver Staining

Silver impregnation produces a range of tints, from the black of nucleoli and the dark brown of intensely stained chromosomal cores, to the golden yellow of interphase nuclei and of the halo that surrounds chromosome cores (see Downes et al. [1994] for color pictures). The technique used is that of Rufas et al. (1987), first used for grasshopper spermatocytes, but here applied for the first time to mammalian somatic chromosomes. Notoriously, reproducible silver staining requires fastidious attention to detail, which we here supply.

Mitotic cells were shaken off, or entire cell populations were removed by trypsinization; cells were fixed in freshly prepared 3:1 methanol-acetic acid, either directly or after 6 min of hypotonic treatment in 50% culture medium in water. This hypotonic treatment apparently does not disturb the metaphase plate or anaphase figures. We have observed that the reproducibility of the technique is greatly reduced when a standard hypotonic treatment in 25% HBSS is used; also, nitrous oxide mitotic arrest is preferable to nocodazole or Colcemid arrest, which more rapidly produce hypercondensed chromosomes that are not easily impregnated with silver.

After three changes of fixative, material was stored overnight at 4°C. Overnight storage generally improves resolution of the chromatin, though recently fixed material can be stained if necessary. Fixed material remains stainable for at least 2 yr.

Fixed material was dropped on to slides and allowed to dry. Dried slides were treated with 2× SSC at 60°C (Sentís et al., 1984). 23-min treatment gives optimum results for Indian muntjac chromosomes; other mammalian cell types may require treatment of from 15 to 30 min. Slides were rinsed in tap water for 5 min, then briefly in distilled water, and air dried.

Silver staining was performed on the same day as 2× SSC pretreatment. The staining solution was prepared immediately before use; 150 μl of 0.05% formic acid in water, freshly prepared, was added to 0.1 g of silver nitrate (Probus or Panreac, Montplet & Esteban S.A., Barcelona, Spain). Larger amounts are not recommended: they tend to precipitate. The mixture was shaken for 30 s to dissolve the silver nitrate. Each slide was given three drops of staining solution, then immediately covered with a coverslip. Slides were put in a wet hot chamber at 75°C. After 3–4 min they were taken out of the chamber and monitored for staining intensity under the microscope. The staining largely stops as the slides cool, but there is still some increase in the background, so once staining was sufficiently intense the coverslips were removed under water and the slides rinsed for 20 s to remove completely the silver solution. Slides were air dried and mounted in DPX mounting medium (BDH Laboratory Supplies, Poole, England).

Analysis of Chromatid Core Lengths

Silver-stained spreads were photographed with a Universal light microscope (Zeiss, Thornwood, NY) and printed at a linear magnification of 3,700. The paths of the stained cores were traced out on transparency sheets. These were then analyzed with a Seescan Solitaire 512 image analyzer (Seescan PLC, Cambridge, UK), so as to measure the total chromatid length and silver-stained core length of complete spread chromosome sets. Reproducibility of length estimate, on repeated analysis of the same spread, was ±3%. 5–10 spreads of identifiable mitotic stages (early prometaphase, late prometaphase, metaphase, hypercondensed metaphase, anaphase, ICRF-193–induced single-core spreads) were analyzed.

Some regions of silver-stained cores appear in photographs to be nearly straight. Others are clearly folded; but we cannot tell whether the path of the core in these regions is a two-dimensional zigzag, a three-dimensional helical coil, or a flattened helix. Direct measurements of core length with the image analyzer assume that the path is two dimensional. However, a simple correction can be made to allow for the possibility that the path is a three-dimensional, regular helix, which seems likely, in view of the evidence for helical core structures (Saitoh and Laemmli, 1994).

Consider a chromosome of length L, of which the core consists of a straight core region of length l_s and a folded zigzag core region of length l_z, containing N side-to-side folded straight elements, each at an angle of pitch θ_1. Then the total axial length of the zigzag region $A = L - l_s$, and so $\theta_1 = \sin^{-1}(A/l_z)$, and the diameter of the zigzag region $D = l_z \cos\theta_1/N$.

But possibly what we see as a two-dimensional zigzag core is really an edge-on projection of a regular helical core, with core length l_h and angle

of pitch θ_2. Then $\theta_2 = \tan^{-1}(A/\pi ND)$, and $l_h = l_z(\sin\theta_1/\sin\theta_2)$, and so the total corrected core length is $l_h + l_s$.

We have calculated the corrected helical core lengths accordingly. Flattened helical cores would obviously be intermediate in length between regular helical and two-dimensional zigzag, depending on the degree of flattening.

We hereafter refer to the nonlinear core structures as *folds* for brevity, without further committing ourselves to the possibilities that they may be helices or zigzags.

Immunolocalization of Topoisomerase IIα

Mitotic cells were collected from control populations and from populations given ICRF-193 (2 µg/ml for 2 h) by shakeoff. After a mild hypotonic treatment (50% of culture medium in water at 37°C for 5 min) they were fixed in -20°C methanol; three changes of the cold methanol followed.

Drops of fixed-cell suspension on coverslips were air dried and then incubated with an IF6 mAb to DNA topoisomerase IIα diluted 1:10 with Dulbecco's PBS for 1 h at 37°C. The antibody used, described by Negri et al. (1993), was raised against full-length human topoisomerase IIα protein; the epitope recognized is not known, but does not lie in the COOH-terminal domain (from Glu_{1178} on) and so must lie in the ATPase or breakage regulation domains (Hickson, I., personal communication).

After washing with PBS, the coverslips were incubated with FITC-conjugated anti-mouse immunoglobulin (Sigma Chemical Co., St. Louis, MO) diluted 1:100 in PBS for 1 h at 37°C. After further washing in PBS, they were treated for 5 min with 1 mg/ml RNAase (DNAase free; Boehringer-Mannheim Biochemicals, Indianapolis, IN) followed by 0.1 µg/ml propidium iodide in PBS for 5 min. After further washing, they were mounted in Vectorshield (Vector Laboratories, Inc., Burlingame, CA) and examined using a confocal microscope (model 600; Bio-Rad Laboratories, Richmond, CA).

Results

Topoisomerase II Inhibition Produces Abnormal Chromosome Cores

We have previously shown that chromosomes forced (by overriding normal G_2 cell cycle controls with caffeine) to attempt condensation from interphase in the presence of topoisomerase II inhibitors form very elongated structures, which, however, have perceptible silver-stained cores (Downes et al., 1994). The G_2 arrest point for topoisomerase II inhibitors is shortly before prophase (Rowley and Kort, 1989); we argued that inhibition of topoisomerase in cells that have passed the arrest point might produce abnormal mitotic structures and so reveal mitotic topoisomerase II activity. We found that once metaphase has been achieved, the topoisomerase II inhibitor ICRF-193 does not interfere with chromosome structure. Metaphase cells treated for 2–4 h with 2 µg/ml ICRF-193, which fully inhibits mitotic topoisomerase II activity (Clarke et al., 1993), show normal, paired, silver-stained chromatid cores and kinetochores (Fig. 1 a).

But by contrast, when unsynchronized cells are given ICRF-193, an entirely new category of silver-stained chromosome appears within 30 min. A single unfolded core runs through the central region, lying between the halos of the sister chromatids (Fig. 1 b); the chromosomes are undercondensed, and the core is sometimes very thin. It appears that, in early mitosis, a topoisomerase-dependent step occurs that is necessary for the formation of the familiar metaphase twin chromatid cores, from a previously unsuspected precursor single core.

The elongated structures seen after treatment with

ICRF-193 are capable of further chromosome condensation and sister chromatid core (SCC) separation, if topoisomerase activity is restored by removal of ICRF-193, while mitotic progression is prevented by nocodazole. After recovery, chromosome organization resembles that in control metaphase cells; condensed SCCs are separated and lie in the middle region of each chromatid (Fig. 1 c). However, core gaps occur, with some recombination events.

After longer times of continuous ICRF-193 treatment of unsynchronized populations, rather different structures appear, with approximately the same chromatid core length as previously; we believe these to be derived from the earlier chromosomes. After 2 h (Fig. 1 d), most chromosomes (78% of a scored sample) still have a single core, such as is seen after shorter periods; some have small patches of separated SCCs (Fig. 1, f and g). With longer incubations, the frequency of this type of structure increases; 58% show separation after 3 h, and some show complete separation of SCCs, with evident core gaps (Fig. 1 h). After 5 h, most fall into this category (Fig. 1 i).

Also, after 3 h, structures appear with longer cores, which are single and with many attenuated regions between more definitely condensed patches of core (Fig. 1 e). We interpret these as derived from cells that had not passed the leaky G_2 topoisomerase II–dependent checkpoint when ICRF-193 was added, but which have subsequently slipped through and are attempting mitosis with extensively concatenated chromatin.

Single and Double Chromosome Cores in Normal Mitosis

If we are correct in supposing that the normal process of mitotic condensation involves a topoisomerase II–dependent separation of a single core, then chromosomes in the early stages of mitosis should reveal this novel structure. We have therefore examined silver-stained mitotic cells from unsynchronized populations. Judged by standard morphological criteria, the structures fall into a consistent sequence. Cells are in prophase until their nucleoli have disappeared; in prometaphase until their kinetochores become intensely stained, well defined, and are arranged on a plate; and in metaphase until their chromatids start to separate. We have analyzed the dimensions of cores in chromosomes at various stages of mitosis; the relative frequencies and dimensions are shown in Table I. Typical structures are shown in Fig. 2.

In prophase chromosomes (Fig. 2 a), there are darkly stained residual nucleolar regions, and the chromatids stain palely. The chromosome core is not distinct from the surrounding halo.

In prometaphase the cores first appear as dark structures at the center of closely associated sister chromatids. The cores run through almost the whole length of the chromosomes, nearly to the tips; they alternate between lengths of double, parallel SCCs and lengths of single core in the midline of the chromosome. The single folded core may be abundant (Fig 2, b and c), or more commonly sparser and interspersed with double regions (Fig. 2 d). The double cores are fairly straight; the single cores, unlike the single cores of ICRF-193–treated material, are prominently
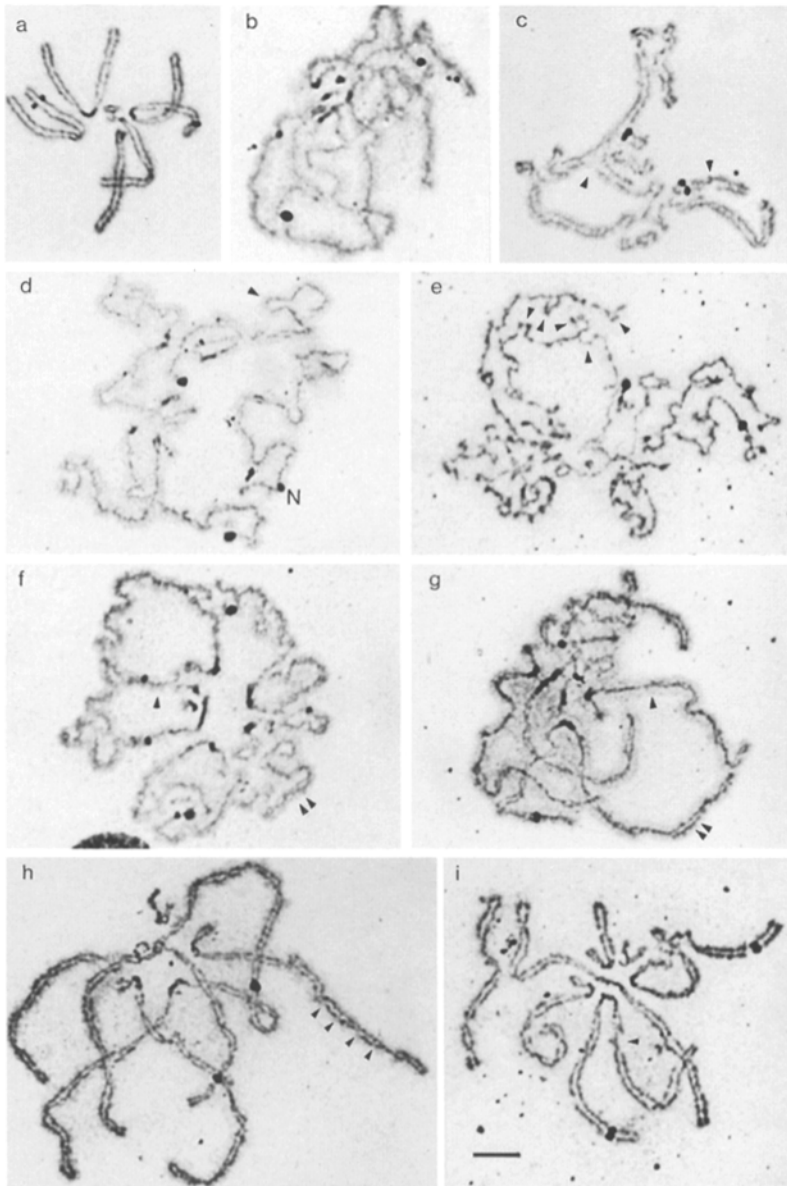
Figure 1. Cores in cells treated with ICRF-193. Silver-stained chromosome spreads from muntjac cells treated with 2 μg/ml ICRF-193 for 0.5–5 h, sometimes with 40 ng/ml nocodazole (*a* and *c*), and either fixed immediately (*a*, *b*, and *d–i*) or after a further period of incubation for 1 h in the presence of nocodazole but absence of ICRF-193 (*c*). (*a*) Chromosomes from cell treated in metaphase with ICRF-193 for 30 min, with double cores and apparently normal metaphase appearance. (*b*) Chromosomes with single unfolded core running through the midline. (*c*) After recovery from ICRF-193, chromosomes show unfolded double chromatid cores; arrows indicate translocations and breaks. (*d*), chromosomes with very thin central single cores, sometimes thrown into loops (*arrow*). (*e*) Elongated chromosomes with single core consisting of attenuated regions between more definitely condensed patches; arrows indicate loops. (*f* and *g*) Chromosomes with mostly single midline cores (*single arrow*) but with patches of double core (*double arrows*). (*h* and *i*) Chromosomes with separated sister cores with multiple core breaks (*arrows*). *N*, nucleolus organizer region. Bar, 5 μm.

folded. These prometaphase structures are obviously related to the ICRF-193–inhibited structures, though the latter are less condensed (compare Fig. 1, *b* and *d* with Fig. 2, *b–d*). However, as Table I shows, the core lengths of ICRF-193–inhibited structures and of prometaphase chromosomes are similar.

Some chromosomes that are still prometaphase (to judge by their kinetochores) have SCCs that are separated along their whole length, except at the folded kinetochore regions (Fig. 2 *e*). SCCs outside kinetochores have an undulating appearance; each SCC runs through the middle of its chromatid. We interpret such spreads as being late prometaphase, derived from the earlier structures by more extensive core separation. These structures are more abundant than those of early prometaphase, which is apparently a very transitory stage.

It appears, from these observations, that the topoisomerase II–dependent separation of chromatid cores is normally completed in prometaphase. However, that is not the end of topoisomerase II–dependent core changes.

In metaphase, the most common mitotic stage, cores are separate and clearly shortened (Fig. 2 *f*). Sister kinetochores tend to be separated, though still closer together than SCCs. Sometimes the kinetochore forms tight folds, thicker than the chromatid core with which it is continuous. When cells are arrested at metaphase for several hours with nitrous oxide, chromosomes become hypercondensed (Fig. 2 *g*), the core fold radius increases, and the number of folds decreases (Table I). It is noticeable that these highly folded, hypercondensed structures are not achieved during prolonged arrest in the presence of ICRF-193 (compare Fig. 1 *a* and Fig. 2 *g*).

During anaphase chromatid segregation, kinetochores are less easy to differentiate from the core. There is an obvious increase in the radius and length of the core fold itself, which now reaches the edges of the chromatids, even at very early stages of anaphase (Fig. 2, *h* and *i*; Table I). This is in contrast to the situation in metaphase chromosomes, in which the core runs in a fold within the central region of the chromatids. We cannot say whether or not

Table I. Frequencies and Dimensions of Mitotic Core and Chromosome Structures in SVM Cells

Stage of mitosis	Frequency of stage	Chromosome length	Core length (2-D spread)	Percentage of core straight	Number of folds	Core length (3-D helix)
	%	μm	μm			μm
ICRF-193 induced	Not applicable	278 ± 18	278 ± 18	100	0	278
Early prometaphase	2.8	131 ± 6	219 ± 11	39	72	288
Late prometaphase	10.8	142 ± 10	196 ± 10	0	106	255
Metaphase	66.1	94 ± 10	116 ± 13	15	81	124
Hypercondensed metaphase	Not applicable	71 ± 3	128 ± 6	8.5	74	180
Anaphase	15.8	91 ± 6	167 ± 12	0	106	238

Stages of mitosis are defined in the text; "ICRF-193 induced" refers to the single-cored figures that appear after ICRF-193 treatment of muntjac cells (Fig. 1, b and d). Frequencies of normal mitotic stages were calculated by scoring 1,000 silver-stained postprophase mitotic spreads from random cells. (The residual 4.5% correspond to telophase figures, in which the cores are too indistinct to measure.) Total lengths of cores and chromosomes per chromosome set were measured as described in Materials and Methods; the mean and standard error of hextuplicate determinations are shown. The fraction of core that is tightly folded was measured, the number of such folds per spread was counted, and the corrected core length, assuming a regular helical coiling of the apparently folded elements, was calculated as described in Materials and Methods.

this anaphase change in core structure is topoisomerase II dependent, because ICRF-193 blocks the transition from metaphase to anaphase (Clarke et al., 1993).

In telophase, the chromosome core becomes more diffusely stained; and with the reconstitution of the daughter nucleus, no cores can be seen (not shown).

Immunolocalization of DNA Topoisomerase IIα

Since topoisomerase II is a core component, and the silver-stained core structure is sensitive to topoisomerase II inhibition, we have investigated whether chromosomes immunostained for topoisomerase IIα resemble silver-stained structures. They do. Control mitotic cells stain in the chromatid cores. In metaphase, SCCs appear separate, running through the middle of each chromatid (Fig. 3, a and b); in anaphase the cores are single (Fig. 3, c and d). Similarly, cells that entered mitosis during treatment with ICRF-193 show immunostaining that resembles the silver staining previously described; commonly, a single core is seen (Fig. 3, e and f), with occasional double regions. Metaphase cells treated for two hours with ICRF-193 showed a similar immunostaining pattern to that of the control cells (not shown).

The Chromosome Core in PCCs

In interphase nuclei, silver impregnation stains nucleoli clearly; other structures, including the kinetochores, are not differentiated. However, core formation can be rapidly induced from interphase cells by fusion with mitotic partners to induce premature condensation; this offers indirect information about core precursors in interphase. Cores are clearly visible in PCCs derived from all stages of interphase, which can be identified by standard morphological criteria (Johnson and Rao, 1970). Those in G_1 are single and lack kinetochores; their cores range from loosely folded through tightly folded to attenuated, depending on the stage of G_1 (Fig. 4, a–c). In S-phase PCCs, the silver-stained structures range from discontinuous fragments to almost fully replicated paired chromatids with double cores (Fig. 4, d–f). G_2 PCCs have distinctive kinetochores; and they always show a well-defined, unfolded core running down each sister chromatid. After short times of PCC induction, G_2 kinetochores and some other regions of the cores remain joined (Fig. 4 g), but complete separation occurs after longer times (Fig. 4 h). This is consistent with SCCs being closely associated dur-

ing interphase, until fusion with a mitotic partner both induces premature condensation and causes the SCCs to separate.

ICRF-193 does not prevent rapid induction of PCCs in interphase cells fused with mitotic partners, even at concentrations that delay unfused cells in G_2 (Downes et al., 1994). The core structure, however, is affected, as would be expected from the action of ICRF-193 on normal chromosome core resolution. Most PCCs induced in the presence of ICRF-193 are very attenuated, with elongated cores (Fig. 5 a). Strikingly, in well-condensed G_2 PCC sets, identified by their kinetochores, there is only a single, midline core (Fig. 5 b).

After PCC formation in the presence of ICRF-193, further condensation can be achieved by restoring topoisomerase II activity, by removing the ICRF-193 and holding cells in mitosis with nocodazole. After such recovery, the induced partner shows a degree of condensation similar to that seen in PCCs induced without ICRF-193. G_2 PCCs seen after this treatment (Fig. 5 c) have double SCCs, with numerous discontinuities and, less frequently, translocations. G_1 PCCs (Fig. 5 d) have single cores.

Discussion

What is the Chromosomal Target for Silver Staining?

Silver staining of chromosomes highlights three different regions: nucleolus organizer (Goodpasture and Bloom, 1975), kinetochore (Denton et al., 1977), and the chromatid core in mitotic chromosomes (Howell and Hsu, 1979) and meiotic chromosomes (Rufas et al., 1983, 1987). In isolated chromosome scaffolds, both the chromatid axes and kinetochores are stained (Earnshaw and Laemmli, 1984; Earnshaw et al., 1985). Though silver nitrate has been used to stain cellular structures for over a century (Ruzicka, 1891; Ramón y Cajal, 1903), the molecular targets are not all established. Silver staining of nucleoli involves thiol-rich regions, mainly in C23 phosphoprotein (Ochs and Busch, 1984); for the other regions, the targets are nonhistone proteins (Jan et al., 1985), probably again those rich in thiols (Gosálvez et al., 1986; de la Torre et al., 1988).

Whatever the core structure that is impregnated by silver may be, it is not detectable before prometaphase. This is not simply a matter of chromatin condensation, which is not less in prophase (Fig. 2 a) than in chromosomes that
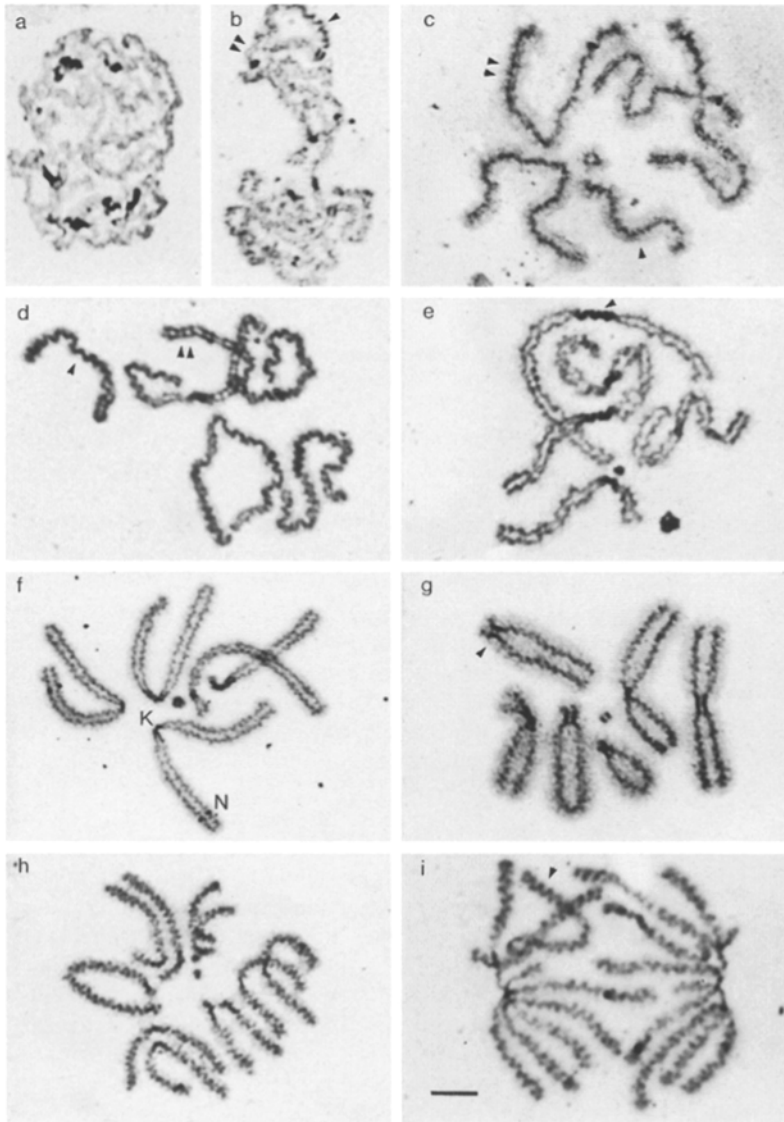
Figure 2. Cores in untreated mitotic cells. Silver-stained chromosome spreads from untreated mitotic cells (*a–f, h* and *i*) or cells arrested for 2 h in high-pressure nitrous oxide (*g*). (*a*) Prophase with residual nucleolar regions stained dark. (*b* and *c*) Early prometaphase; core running along the center of chromatin halo has single folded regions (*single arrow*) alternating with straighter double regions (*double arrows*); nucleoli are absent. (*d*) Late prometaphase with some single cores (*arrow*) and extensive regions of double cores (*double arrows*), often with transverse connections between the parallel cores. (*e*) Late prometaphase with double cores except in single, folded kinetochore regions (*arrow*). (*f*) Metaphase with kinetochores arranged on metaphase plate. (*g*) Hypercondensed metaphase with wide chromatin halos around central cores in each chromatid; arrow indicates kinetochore. (*h*) Early anaphase with slight separation between sister kinetochores. (*i*) Late anaphase; arrow indicates region of folding of core. *K*, kinetochore; *N*, nucleolus organizer region. Bar, 5 μm.

arrive at metaphase in the presence of topoisomerase II inhibitors (Fig. 1, *b* and *d–i*) or in PCCs formed in the presence of ICRF-193 (Fig. 5, *a* and *b*). Likewise, we have been unable to detect kinetochore silver staining in interphase despite the advantage of using a cell line with a low chromosome number and remarkably large kinetochores.

Possible candidates for the silver-staining core protein include the recently sequenced scaffold protein ScII, which occurs in isolated mitotic scaffolds but is not detected in nuclear matrix and is preferentially stained by antibodies in the chromosome core region (Saitoh et al., 1994); or the related heterodimeric coiled-coil proteins XCAP-C and XCAP-E, which likewise are detected in chromatin only in mitosis, bind to the chromatid axis, and are required for chromosome condensation (Hirano and Mitchison, 1994). Since ScII binds to the predominant scaffold protein, topoisomerase II (Ma et al., 1993), staining of ScII would indirectly localize topoisomerase II, or at least a fraction of it.

How far topoisomerase II is localized in a central chromosomal region is uncertain. Immunostaining shows its occurrence (not necessarily as a continuous structure) in the core of dehistonized material (Earnshaw and Heck, 1985) and fixed intact chromosomes (Earnshaw et al., 1985; Saitoh and Laemmli, 1994; Fig. 3), but there are other reports of it being diffusely distributed throughout entire chromosomes (Negri et al., 1992; Hirano and Mitchison, 1993; Swedlow et al., 1993). These are, admittedly, from embryonic material where topoisomerase II is present in great excess; but a peripheral position for some molecules of topoisomerase II fits the need for topoisomerase II activity in the final stages of chromatid segregation (Downes et al., 1991; Shamu and Murray, 1992; Clarke et al., 1993). An exclusively central location for the enzyme is not obviously consistent with the separation of loops of DNA originating from different sister chromatids. Possibly the peripheral molecules of topoisomerase II are lost during some procedures of extraction or fixation. It is noteworthy that the antibody MPM-2, which recognizes a phosphorylated epitope in mitotic cells and predominantly binds a phosphorylated form of topoisomerase IIa (Taagepara et al., 1993), stains a chromosomal core region both

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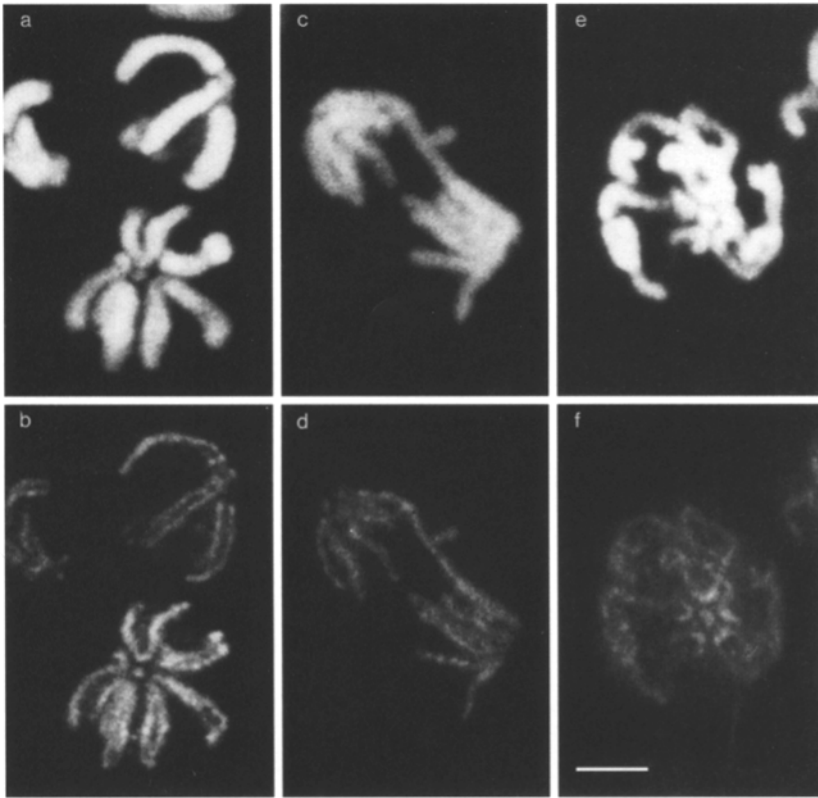
Figure 3. Immunolocalization of topoisomerase IIα. (*a–d*) Control mitotic cells. (*e* and *f*) Cells treated with ICRF-193. Pairs of confocal microscope pictures of cells stained with mouse antibody to topoisomerase IIα followed by FITC-conjugated anti–mouse immunoglobulin (*b, d,* and *f*) and counterstained with propidium iodide (*a, c,* and *e*). Each picture is a projected series of three 1 μm optical sections. Bar, 5 μm.

in conditions where polyclonal antibodies to topoisomerase II stain the whole chromosome (Hirano and Mitchison, 1991), and also after most of the topoisomerase II has been extracted (Hirano and Mitchison, 1993).

A minimal conclusion, given the effect of inhibitors of topoisomerase II on the silver-stained core, would be that some part of the chromosomal content of topoisomerase II interacts with the material that becomes impregnated with silver.

A Unique Form of Chromosome Organization in Early Prometaphase

Whatever its target, silver staining has revealed two different kinds of chromosome organization. One corresponds to the standard model of chromatin organization during mitosis; each chromatid has a central core, which is further compacted by folding. Such cores are seen by silver staining from metaphase to the end of mitosis (Howell and Hsu, 1979).

However, the structures we observe in early prometaphase clearly show a novel kind of organization. Some regions are related to metaphase-type mitotic organization, with two central chromatid cores, which are not, however, folded; others show a single folded core, in the midline of the chromosome, in which sister chromatid elements cannot be distinguished. At the boundaries between the two kinds of regions, there is no evidence of intertwining of the emergent twin cores; we therefore suggest that if the core is helically coiled, then the coiling of the sister elements of the single midline core is paranemic. The existence of a single midline core may imply a lateral position of the

chromatid core within each sister chromatid, changing to a central position in the standard mitotic structure.

This single-cored element of early prometaphase structure is entirely unexpected; but its existence must be allowed for in any model of chromosome condensation.

Topoisomerase II Activity and Chromatid Core Structure

The resolution of the early prometaphase single core into paired parallel cores is sensitive to the inhibitor ICRF-193, and therefore appears to involve DNA topoisomerase II. Treatment of cells in prophase or very late G_2 with ICRF-193 allows the formation of a single midline chromosome core, but not (initially) the conversion of this core to two separate mitotic chromatid cores. Likewise, G_2 cells driven into PCCs form separate chromatid cores, except in the presence of ICRF-193. Since the separation of the two SCCs that combine to make the central core is dependent on topoisomerase II function, though the formation of the cores is not, we suppose that core separation depends on the decatenatory activity of topoisomerase II. The possibility that ICRF-193 inhibits some other function of topoisomerase II cannot be formally excluded; but since there is no evidence for other functions, we will continue on the hypothesis that topoisomerase II function involves decatenation. If this is so, then the precursors of the prometaphase core must in G2 cells be connected by concatenated DNA loops, which have not yet been separated after replication.

It seems unlikely that such catenations, connecting central core regions or their precursors, could involve more
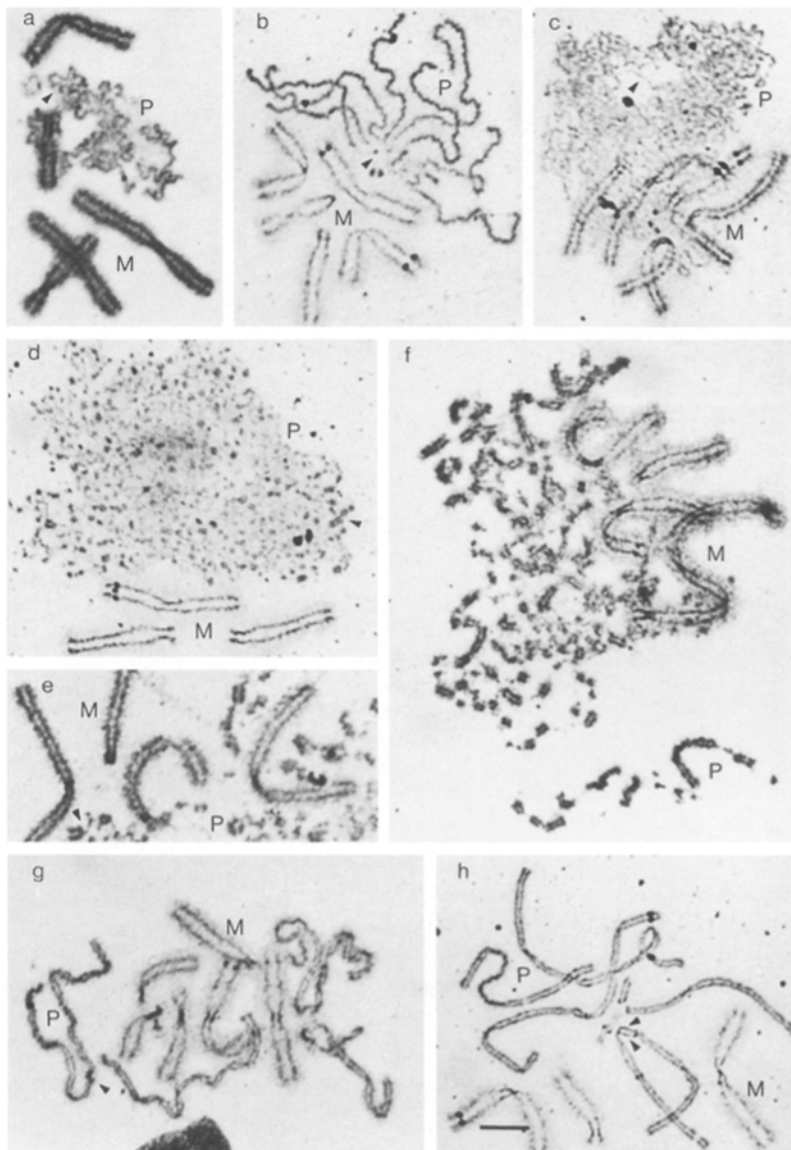
Figure 4. Cores in PCCs. Silver-stained spreads of mitotic chromosomes (*M*) and PCCs (*P*) in cells induced for 1 h (*a–f*, and *h*) or 0.5 h (*g*) by fusion between mitotic and interphase muntjac cells. (*a*) Early G_1 PCCs; arrow indicates folded region. (*b*) Later G_1 PCCs; note orientation of G_1 chromosomes on metaphase plate (*arrow*). (*c*) Late G_1 PCCs with continuous (*arrow*) and discontinuous cores. (*d*) Early S-phase PCCs with double replicated regions (*arrow*). (*e*) Mid S-phase PCCs with partially replicated regions (*arrow*). (*f*) Late S-phase PCCs; note partially replicated chromosome at bottom. (*g*) G_2 PCCs at early stage of induction with incompletely separated sister cores; arrow indicates darkly stained kinetochore. (*h*) G_2 PCCs at later stage of induction, with separated cores; arrows indicate kinetochore. Bar, 5 μm.

than a small fraction of the DNA. In mammalian G_2 nuclei, replicated sequences appear as two closely spaced, but distinguishable, signals in FISH mapping (fluorescence in situ hybridization; Lawrence et al., 1990). However, in G_2 PCCs after brief periods of induction, regions of single core like those of normal prometaphase appear (Fig. 4 *g*), and G_2 cores are uniformly single in PCCs induced with inhibited topoisomerase II (Fig. 5 *b*). This suggests that, in interphase cells, from the moment of replication, the chromatin regions bound to the nuclear matrix—the putative precursors of the core—are in a partially catenated stage, and remain there until prometaphase. Some catenations connecting sister chromatids survive until the metaphase–anaphase transition, to judge by the sensitivity of that process to topoisomerase II inhibitors and mutations; but if any of these remnant catenations connect sister cores, they must do so very loosely.

Previous work has shown that topoisomerase II is in some way essential for chromosome condensation (Ue-

mura et al., 1987; Newport and Spann, 1987; Wood and Earnshaw, 1990; Adachi et al., 1991; Hirano and Mitchison, 1991; Gorbsky, 1994; Downes et al., 1994). This work is both the first demonstration that one of the necessary functions is to separate chromatid cores, and the first indication that such a step does not normally occur till after prophase.

ICRF-193 inhibition of core separation is not permanent. The single-cored structures generated in the presence of ICRF-193, after spending at least two further hours arrested in metaphase, start to separate their SCCs; the newly separated cores display, however, a large number of discontinuities. Complete chromatid breakage is likewise produced by attempts to achieve segregation in the absence of decatenatory activity (Holm et al., 1989). We conclude that resolution of the initial single core involves powerful molecular forces, sufficient to break the core-connecting catenations where necessary.

As well as being required for core separation, topo-
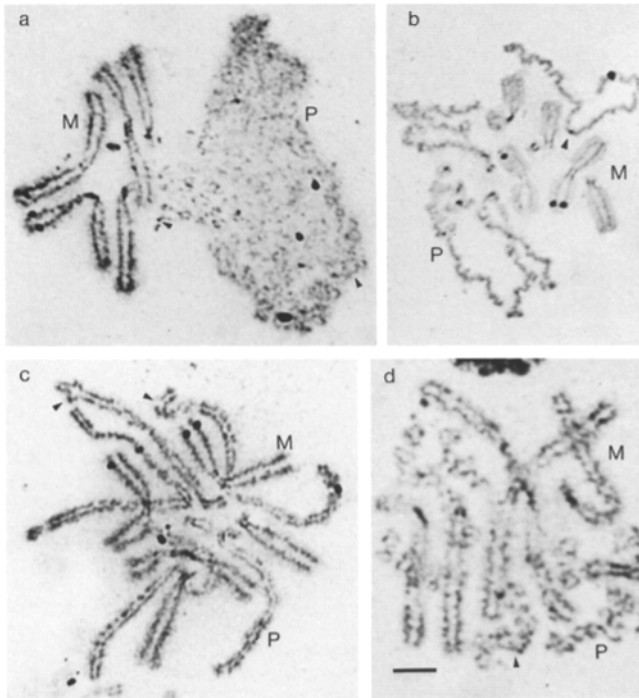
Figure 5. Cores in PCCs induced in the presence of ICRF-193. Silver-stained spreads of mitotic chromosomes (*M*) and PCCs (*P*) in cells induced by fusions between mitotic and interphase muntjac cells in the presence of ICRF-193, and fixed either immediately (*a* and *b*) or after removal of ICRF-193 and further incubation for 1 h with nocodazole (*c* and *d*). (*a*) Threadlike PCCs with possible kinetochore (*arrow*) at the metaphase plate. (*b*) G_2 PCCs with clear kinetochores (*arrow*) and single chromatid core. (*c*) G_2 PCCs with separated SCCs after recovery from ICRF-193 treatment with breaks and translocations (*arrows*). (*d*) G_1 PCCs (*arrow*) among mitotic chromosomes from the fusion partner. Bar, 5 μm.

isomerase II also plays a part in the folding of the prometaphase single core; folded single cores cannot be achieved in the presence of ICRF-193. After prometaphase, the core dimensions and topology continue to change, and at least some of these changes are also dependent on topoisomerase II activity. We cannot tell whether or not the shortening and folding of the paired chromatid axes that occurs between prometaphase and metaphase is topoisomerase dependent. Since metaphase core staining is not affected by ICRF-193 (Fig. 1 *a*), core integrity does not depend on continued topoisomerase activity; comparably, isolated chromosome structure is not affected by removal of topoisomerase II (Hirano and Mitchison, 1993). But inhibition of topoisomerase II, as well as preventing the prometaphase core separation, prevents subsequent changes in the folding of chromatid cores. There is a clear ICRF-193–sensitive reduction in chromatid length, but not core length, that occurs during prolonged metaphase arrest. There is also a change in core folding on passing from metaphase to anaphase; it is tempting to regard this as a consequence of the release of the various factors, including catenated sister loops that are resolved by topoisomerase II, and that hold sister chromatids in register until the metaphase–anaphase transition (Holm, 1994).

Chromosome Organization during the Somatic Cell Cycle

A model for chromosome organization during the somatic cell cycle is shown in Fig. 6. There are two alternating phases: a catenated-core stage, in which SCCs (or precursors of cores in interphase) are united; and a noncatenated-core stage, in which separate sister chromatid cores appear in the axial region of each chromatid (or, in G_1 cells, each core precursor is separate). The transition from the decatenated to the catenated stage occurs at replication; we infer from the effects of ICRF-193, and from the
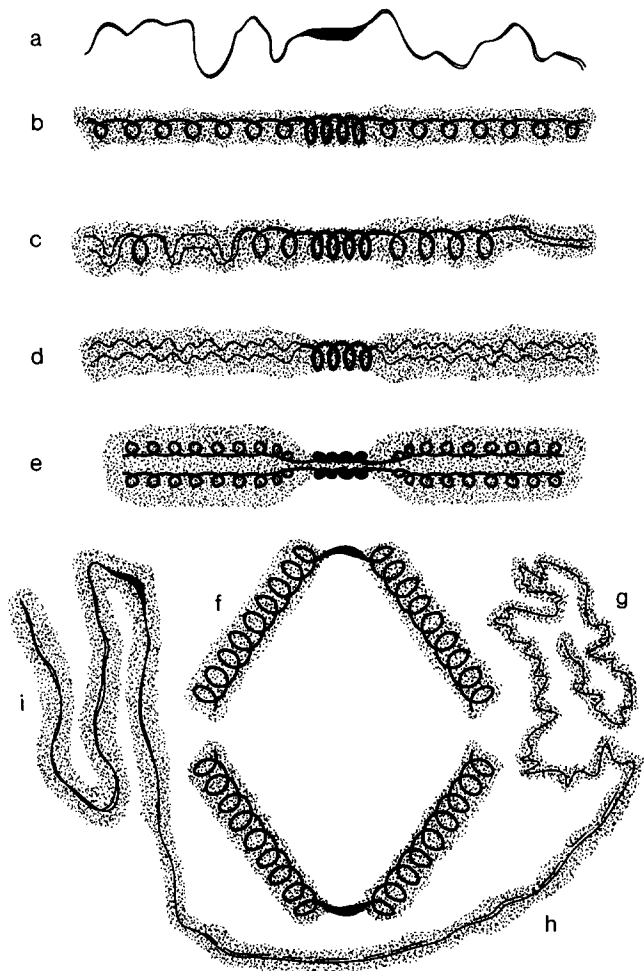
Figure 6. Model for changes in chromosome cores through the cell cycle. (*a*) Hypothetical postreplicative premitotic structure (never visualized by silver staining) with closely associated uncondensed cores. (*b*) Presumptive state at prophase–prometaphase boundary with closely associated folded cores. (*c*) Earliest visualized prometaphase with regions of single folded core interspersed with regions of double-parallel core. (*d*) Late prometaphase with sister cores entirely separate and parallel except at the kinetochore. (*e*) Metaphase with folded cores of narrow diameter (central in chromatid); sister kinetochores are separate but closely associated. (*f*) Anaphase with sister chromatid cores completely separated; folds of core extend to perimeter of chromatids. (*g–i*) Prematurely condensed chromosome depicted with three regions corresponding respectively to G_1 with unfolded but undulating core (*g*), S-phase with discontinuous double patches (*h*), and G_2 with double cores (*i*). See Discussion for further details.

PCC studies, that this produces a topologically continuous single-core precursor (Fig. 6 *a*), which cannot be detected as a silver-stained core. Since no core is detectable until the end of prophase, we assume that either the earlier catenated structures do not involve tight apposition of core elements, or they lack an argyrophilic component (perhaps because this component is not synthesized until just before the start of mitosis).

We hypothesize that if the core were seen at the very first moment of prometaphase, it would be like that shown in Fig. 6 *b*: a continuous, single, folded structure. But such a structure must be extremely transient, before decatenation and separation of the folded single core into twin unfolded cores (Fig. 6, *c* and *d*) begin in early prometaphase, corresponding to a change from lateral to central placing of the core element in the chromatid. Metaphase (Fig. 6 *e*) involves efforts to achieve maximum chromosome condensation, after reaching the decatenated stage, by compacting and folding the chromosome cores. After anaphase segregation has taken place, an expansion and lengthening of the folded core is a prelude to eventual decondensation.

DNA topoisomerase II plays a basic role in this chromosome organization cycle. We cannot say whether the topoisomerase-dependent core shortening involves a structural role for topoisomerase II in the packing and assembly of the core, or a need to remove residual decatenations, possibly in the peripheral chromatin regions. But the separation of the prometaphase catenated single core is obviously topoisomerase dependent.

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