Cell Cycle Control of Higher-Order Chromatin Assembly around Naked DNA In Vitro

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Abstract. We have developed an in vitro system in which higher-order chromatin structures are assembled around naked DNAs in a cell cycle-dependent manner. Membrane-free soluble extracts specific to interphase and mitotic states were prepared from Xenopus eggs. When high molecular weight DNA is incubated with interphase extracts, fluffy chromatin-like structures are assembled. In contrast, mitotic extracts produce highly condensed chromosome-like structures. Immunofluorescence studies show that a monoclonal antibody MPM-2, which recognizes a class of mitosis-specific phosphoproteins, stains the "core" or "axis" of condensed mitotic chromatin but not interphase chromatin. By adding mitotic extracts, interphase chromatin structures are synchronously converted into the condensed state. The increasingly condensed state of chromatin correlates with the appearance and structural rearrangements of the MPM-2-stained structures. These results suggest that mitosis-specific phosphoproteins recognized by MPM-2 may be directly involved in the assembly of the chromosome scaffold-like structures and chromatin condensation. Although both extracts promote nucleosome assembly at the same rate, topoisomerase II (topo II) activity is four to five times higher in mitotic extracts compared with interphase extracts. The addition of a topo II inhibitor VM-26 into mitotic assembly mixtures disturbs the organization of the MPM-2-stained structures and affects the final stage of chromatin condensation. This in vitro system should be useful for identifying cis- and trans-acting elements responsible for higher-order chromatin assembly and its structural changes in the cell cycle.

Eukaryotic cells exhibit dramatic changes of chromatin structure in the cell cycle. Faithful structural rearrangements of higher-order chromatin are thought to be required for a variety of nuclear functions including DNA replication, transcription, and chromosome segregation. Organization of higher-order chromatin is achieved by successive levels of folding of DNA molecules. The genomic DNA is assembled into nucleosome filaments and further packed into the 30-nm fibers. Histone H1 is known to be required for the formation of 30-nm fiber (Thoma et al., 1979) and phosphorylation of histone H1 and H3 has been shown to correlate with chromatin condensation in mitosis (Gurley et al., 1978; Matthews and Bradbury, 1978). The 30-nm fibers are thought to be organized into chromatin loop domains of 50-100 kb in both interphase nuclei and mitotic chromosomes. The network of nonhistone proteins that is postulated to anchor the chromatin loops is called the nuclear and chromosome scaffold (reviewed by Gasser et al., 1989). One of major chromosome scaffold proteins has been identified as DNA topoisomerase II (topo II; Earnshaw et al., 1985; Gasser et al., 1986), suggesting that topo II might function both catalytically in regulating the DNA conformation and structurally in maintaining chromosome organization.

DNA sequences that preferentially bind to the mitotic chromosome scaffold and to the interphase nuclear matrix have been identified and characterized in several organisms from yeast to human (Mirkovitch et al., 1984; Cockerill and Garrard, 1986; Amati and Gasser, 1988). These sequences called scaffold-associated region or matrix-associated region contain clusters of AT-rich motifs that are related to the topo II cleavage consensus sequence. Although scaffold- or matrix-associated regions are proposed to define the base of the chromatin loops (reviewed by Gasser et al., 1989), their actual role in vivo is not clear.

Genetic experiments in yeasts have demonstrated in vivo functions of some of these chromatin components. For example, deletion mutations in histone H4, a component of nucleosomes, lead to transcriptional derepression of the silent mating loci, clearly suggesting the importance of chromatin structure in gene expression (Kayne et al., 1988). Topo II has been shown to play an essential role in chromosome condensation as well as segregation in mitosis (e.g., Uemura et al., 1987). Furthermore, several gene products involved in gene regulation and chromosome segregation have been characterized as possible candidates for nuclear scaffold components (Hirano et al., 1988, 1990; Hofmann et al., 1989).

Despite such recent progress in our understanding of individual chromatin components, molecular mechanisms responsible for the assembly of higher-order chromatin and its regulation in the cell cycle are poorly understood. One approach to address this problem is to reconstitute higher-order chromatin structure around naked DNA in vitro. An ideal system for such studies is provided by Xenopus egg ex-
tracts, which were used for studying nucleosome assembly in the pioneering experiments of Laskey et al. (1977). Although similar extracts have also been used to study DNA replication (Blow and Laskey, 1986) and nuclear envelope assembly and breakdown (Newport, 1987; Newport and Spann, 1987), the assembly of higher-order chromatin structures around naked DNA has not yet been well characterized. On the other hand, recent elucidation of regulatory mechanisms of the cell cycle has made it possible to prepare more reliable extracts in which the cell cycle state can be easily manipulated in vitro (Lohka and Maller, 1985; Murray and Kirschner, 1989; Murray et al., 1989), and to reconstitute a variety of cellular events in a cell cycle-dependent manner in vitro: these include initiation of DNA replication (Blow and Sleeman, 1990), microtubule dynamics (Belmont et al., 1990; Verde et al., 1990; Vale, 1991), mitotic spindle assembly (Sawin and Mitchison, 199la,b), and membrane transport (Allan and Vale, 1991).

In this paper, we describe an in vitro system for studying higher-order chromatin assembly around naked DNAs using cell cycle-specific Xenopus egg extracts. We have found that interphase extracts support the assembly of fluffy chromatin-like structures, whereas mitotic extracts produce highly condensed chromosome-like structures. We present evidence that the condensed state of chromatin correlates with the assembly of the chromosome scaffold-like structures that are recognized by a mitosis-specific monoclonal antibody MPM-2.

Materials and Methods

Preparation of Cell Cycle-specific Extracts

Mitotic crude extracts were prepared from unfertilized eggs of Xenopus laevis according to Murray et al. (1989). To prepare interphase crude extracts, mitotic crude extracts were activated in vitro by adding 1:100 vol of 40 mM CaCl₂, 100 mM KCl, 1 mM MgCl₂, and 1:100 vol of 10 mg/ml cycloheximide. After incubation for 30 min at 20°C, 1:100 vol of 40 mM EGTA was added and the mixture was transferred to ice. Alternatively, interphase crude extracts were prepared from electrically activated eggs as described (Murray and Kirschner, 1989) with the exception that cycloheximide (100 µg/ml) was added at the breakage stage of eggs. These extracts were induced to enter mitosis by incubating for 30 min at 20°C with a bac- terially expressed V-ATPase (100 µM), a nondegradable sea urchin cyclin B lacking the NH₂-terminal 90 amino acids (Murray et al., 1989). To make soluble extracts, crude interphase and mitotic extracts were centrifuged at 50,000 rpm for 3 h at 4°C (model TLS55; Beckman Instruments, Inc., Fullerton, CA) and the clear central layer was carefully collected using a 20-gauge needle. These extracts were divided into 50-µl aliquots and frozen in liquid nitrogen. As reported by many other groups (e.g., Allan and Vale, 1991), we found that mitotic extracts prepared from unfertilized eggs or converted from interphase extracts in vitro behaved almost identically. Similarly, interphase extracts prepared from activated eggs or converted from mitotic extracts in vitro produced essentially the same results. Most of the experiments described in the text were performed using interphase and mitotic extracts prepared by the first method. To confirm the cell cycle regulation, extracts were also prepared by the second method and used in some experiments. Morphological activities of higher-order chromatin assembly varied slightly between different batches of extracts. To get reproducible results, all morphological experiments were performed after checking the two biochemical activities of extracts, efficient nucleosome assembly and cell cycle-dependent topo II activity (see below).

Template DNAs

High molecular weight Xenopus genomic DNA was isolated from female liver as described (Sambrook et al., 1989). Average length of DNA was ~100-200 kb as judged by 0.3% agarose electrophoresis with λ DNA ladder as a standard.

Chromatin Assembly Reactions

DNA was added to 50 µl of soluble interphase or mitotic extracts at a final concentration of 10 µg/ml and incubated at 20°C. To monitor chromatin assembly, aliquots of the reaction mixture were taken and mixed with an equal volume of 50% (wt/vol) glycerol, 10% formaldehyde, and 1 µg/ml Hoechst 33258 in MMR (100 mM NaCl, 2 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 0.1 mM EGTA, and 5 mM HEPES [pH 7.8]) and observed by fluorescent microscopy (Photoscope III; Carl Zeiss, Inc., Thornwood, NY). The DNA content of a single mitotic chromatin structure (100-200 DNA molecules/structure) was roughly estimated based on the average length of a single DNA molecule (100-200 kb), the concentration of DNA added into extracts (10 µg/ml) and that of chromatin structures assembled in extracts (~3-10 x 10⁶ structures/ml). The final packing ratio of DNA was obtained from the observed length of rod-shaped chromatin bodies and the total DNA length making a single chromatin structure. Although the concentration of assembled structures slightly varies between different batches of extracts, final packing ratio of DNA in highly condensed bodies was consistently estimated to be between 1,000 and 5,000. We also estimated that a single fluffy chromatin structure assembled in interphase extracts contains similar number of DNA molecules (100-200) although we noted the estimation more difficult because of their heterogeneity in size and shape. VM-26 (4'-demethylpodophyllotoxin thenylidine-β-D-glucoside; a generous gift from Bristol-Meyers) was dissolved in DMSO as a stock solution of 10 mM. The final DMSO concentration of up to 0.4% did not affect assembly reactions.

Immunofluorescence

For immunofluorescent staining, 10 µl of assembly mixture was fixed by the addition of 200 µl of 1% formaldehyde in XB-EGTA (100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 5 mM EGTA, and 10 mM HEPES [pH 7.7] and 50 mM sucrose) and incubated for 15 min at room temperature. The mixture was layered over a cushion of 30% glycerol in XB-EGTA, sedimented onto a coverslip, and postfixed in −20°C methanol. The coverslip was then stained with a mouse monoclonal antibody MPM2 (Davis et al., 1987) followed by fluorescein-conjugated goat anti-mouse IgG and counterstained with Hoechst 33258. CHO chromosomes were isolated as described (Mithchison and Kirschner, 1985; a gift from Dr. A. A. Hyman). Chromosomes were fixed with 1% formaldehyde in PME (10 mM PIPES [pH 6.9], 5 mM MgCl₂, and 1 mM EGTA), sedimented onto a coverslip, and stained as above.

Nucleosome Assembly and Topo II Assay

Nucleosome assembly was assayed as described by Dilworth et al. (1987) with minor modifications. Supercoiled pGEM2 plasmid DNA (Promega Corp., Madison, WI) was treated with calf thymus topoisomerase I (Bethesda Research Laboratories, Gaithersburg, MD) and deproteinized by phenol extraction to yield relaxed DNA. DNA was added to extracts at a final concentration of 10 µg/ml and incubated at 20°C. At appropriate intervals, aliquots were taken and the reactions were terminated by adding 20 vol of stop solution (20 mM Tris.HCl [pH 8.0], 20 mM EDTA, 0.5% SDS, and 500 µg/ml proteinase K). After incubation of the mixture at 37°C for 60 min, the reaction mixture was deproteinized with phenol and the DNA was precipitated with ethanol. After centrifugation the pellet DNA was dissolved in 10 µl of TE (10 mM Tris.HCl [pH 8.0] and 1 mM EDTA) containing 50 µg/ml RNase A and incubated at 37°C for 15 min. DNA was electrophoresed on a 1.0% agarose gel and visualized by staining with ethidium bromide. For topo II-mediated DNA cleavage assay, increasing concentration of VM26 (4'-demethylpodophyllotoxin thenylidine-β-D-glucoside; a generous gift from Bristol-Meyers, Syrracuse, NY) was added to extracts immediately before the addition of DNA. After 3 h incubation at 20°C, the reaction was stopped and analyzed as above.

Results

Higher-Order Chromatin Assembly around Naked DNA in Interphase and Mitotic Extracts

Although it has been demonstrated that in Xenopus egg extracts naked DNA acts as a template for nucleosome assembly (Laskey et al., 1977; reviewed by Laskey and Leno, 1990), DNA replication (Blow and Laskey, 1986), and nuclear envelope assembly (Newport, 1987), the assembly of
higher-order chromatin structures has not been well characterized. Newport (1987) reported that, after nucleosome assembly around DNA, chromatin structures became resistant to extraction by the combined treatment of nonionic detergent and high salt, implicating the possible formation of scaffold. This observation prompted us to investigate whether some kind of higher-order chromatin structures are indeed assembled around naked DNA in vitro and whether the assembly reactions are regulated in the cell cycle. We prepared extracts specific to interphase and mitotic states from Xenopus eggs. To reduce the complexity contributed by the interaction between chromatin and nuclear envelope components, membrane-free soluble extracts were prepared by high speed centrifugation and used for the following experiments.

When high molecular weight Xenopus genomic DNA was added to interphase extracts, a series of morphological rearrangements was observed. Immediately after the addition, DNA was visible as long, thinfilaments. Between 30 and 60 min, the filaments started to be folded and partially condensed. After 2-3 h, fluffy chromatin-like structures were observed (Fig. 1 a). We have observed no indication of further condensation after longer incubation. Instead, the fluffy structures tended to be damaged and dispersed after 5-6 h. In mitotic extracts, DNA showed completely different structural changes. In the first hour, no obvious structures were visible. Between 1 and 2 h, tiny dot structures (~0.2 μm in diameter) appeared. The dots were then associated with each other to form brightly stained chromatin bodies (0.5-1 μm in diameter). By 3 h incubation, the association proceeded and rod-shaped chromosome-like structures were observed (Fig. 1 b). The shape and the intensity of staining resembled those of mitotic condensed chromosomes. The diameter and length of typical rod structures were 1-2 and 2-5 μm, respectively. Based on the concentration of DNA added into extracts and that of the assembled structures, we roughly estimate that a single structure consists of 100-200 molecules of DNA (a single DNA molecule is ~100-200 kb long) and the final packing ratio of DNA is between 1,000 and 5,000. (An interphase chromatin structure also contains the same order of DNA molecules; see Materials and Methods). As shown in Fig. 1, condensed chromatin bodies assembled in mitotic extracts were more fluorescent than fluffy chromatin masses assembled in interphase extracts, indicating a higher concentration of DNA per unit volume, and thus a higher packing ratio. To reflect the difference in the condensation states of these two structures, we call the fluffy interphase chromatin “decondensed” chromatin despite its compaction relative to naked DNA. In contrast to interphase chromatin masses, highly condensed mitotic chromatin structures appeared stable: no significant structural changes were observed after longer incubation (at least 6 h). When the mitotic assembly mixture was treated with 2 M KCI, DNA formed an expanded halo around a central core of mitotic chromatin, suggesting the existence of a substructure that is resistant to high salt extraction. The same treatment of interphase assembly mixture caused complete disruption and dispersion of the fluffy structures. Neither interphase nor mitotic chromatin structures were stained with anti-Xenopus lamin A antibody (Benavente et al., 1985; data not shown).

**MPM-2 Stains the “Core” of Mitotic Chromatin but Not Interphase Chromatin**

Since many mitotic events are controlled by phosphorylation, we wished to test whether the condensation of mitotic chromatin in our system is mediated by mitosis-specific phosphorylation. To address this question, chromatin structures were assembled in interphase and mitotic extracts and stained by a monoclonal antibody MPM-2 which recognizes a class of mitosis-specific phosphoproteins (Davis et al., 1983; Vandré et al., 1984, 1986). MPM-2 did not stain decondensed chromatin masses assembled in interphase extracts (Fig. 2, a and b). In contrast, condensed chromatin bodies assembled in mitotic extracts were strongly stained with MPM-2. The staining was not uniform and discrete internal structures were observed as dots or fibers in the center of chromatin bodies (Fig. 2 c-h). The staining of peripheral regions was, if any, very weak. We considered the possibility that MPM-2 in fact stains interphase structures, but even long exposures of fields like Fig. 2 b failed to give any fluorescence signal over background. Thus, we are confident that the MPM-2 epitope is indeed mitosis-specific though the protein being recognized may well be present on interphase chromatin.

To test whether the chromatin assembly described above
is specific to Xenopus high molecular weight DNA, noneukaryotic DNA was used as a template. When λ DNA was incubated with interphase extracts, decondensed fluffy structures were assembled (Fig. 3a). The shape and the staining intensity were very similar to those assembled around Xenopus DNA. Mitotic extracts, again, produced highly condensed structures around λ DNA (Fig. 3c). MPM-2 stained mitotic chromatin bodies (Fig. 3d) but not interphase chromatin masses (Fig. 3b). In highly condensed structures assembled around λ DNA, axial staining was predominant and branched structures were often observed (Fig. 3d). These results suggest that specific eukaryotic DNA sequences are not required for the higher-order chromatin assembly in vitro.

Chromatin Condensation in an “Interphase-to-Mitotic” Assembly Pathway

The results described above have clearly shown that interphase and mitotic extracts exhibited the two distinct pathways in higher-order chromatin assembly around naked DNAs. In vivo, interphase chromatin is the template from which highly condensed mitotic chromosomes are assembled. To investigate this physiological pathway of chromatin condensation in vitro, we attempted to convert interphase chromatin into condensed mitotic chromatin. This was achieved by the two-step addition of interphase and mitotic extracts (Sawin and Mitchison, 1991a). High molecular weight Xenopus genomic DNA was first added into interphase extracts to form fluffy chromatin masses (Fig. 4a). The assembly mixture was then driven into the mitotic state by the addition of 2 vol of mitotic extract. Immediately after the addition of mitotic extracts the fluffy decondensed structures started to show a series of morphological changes. At 30 min they were folded and formed partially condensed structures (Fig. 4c). As the condensation proceeded, the intensity of staining with the DNA binding dye increased (Fig. 4, c and e). By 120 min all chromatin structures were converted into the highly condensed state (Fig. 4, g and i). This structural transformation occurred in a highly synchronous manner: at most time points all chromatin struc-
The axial structure stained with MPM-2 is reminiscent of the chromosome scaffold that is implicated in mitotic chromosome assembly and organization (Paulson and Laemmli, 1977). For this reason, we investigated the localization of MPM-2 antigens in mitotic chromosomes isolated from CHO cells. It has been shown that, in permeabilized mitotic cells, kinetochores are strongly stained with MPM-2 (Vander et al., 1984, 1986). Consistent with these previous reports, we observed the strong staining of kinetochores that were seen as a pair of dots on the primary constriction of an isolated chromosome (Fig. 5, a and b). In addition to the kinetochore staining, we found that MPM-2 stained the central axial region of chromosomes, extending along the entire length of sister chromatids. The staining pattern was clearly distinct from the distribution of chromosomal DNA. In a minor population of chromosomes (~20%), the axial staining was more intense and dominant over the kinetochore staining (Fig. 5, c and d). Although these two populations of chromosomes were indistinguishable by their shapes, the different MPM-2 staining patterns may result from the slight difference in the mitotic stages at which they were arrested or from the changes of phosphorylation state during isolation.

**Nucleosome Assembly and Topo II Activity in Interphase and Mitotic Extracts**

To examine the assembly processes of chromatin at a molecular level, kinetics of nucleosome assembly were compared in interphase and mitotic extracts. Since the assembly of each nucleosome introduces one superhelical turn and the resulting superhelical tension is removed by the endogenous topoisomerases, the extent of assembly can be measured by determining the average number of superhelical turns in a circular plasmid DNA after deproteinization (Laskey et al., 1977). Relaxed plasmid DNA was added to interphase and mitotic extracts, and a time course of nucleosome assembly was followed. As shown in Fig. 6 a, all relaxed plasmids were converted into the supercoiled form after 1 h incubation. The rates of nucleosome assembly were indistinguishable between interphase and mitotic extracts. Micrococcal nuclease digestion produced the same pattern of a nucleosome ladder containing four to five discrete bands spaced ~180 bp in both extracts (data not shown). These results indicate that the different condensation states of chromatins structures assembled in interphase and mitotic extracts are produced by the subsequent levels of folding processes after nucleosome assembly.

To investigate a possible role of topo II activity in higher-order chromatin assembly in vitro, we compared the activity of topo II in interphase and mitotic extracts. To measure the topo II activity using the same template used for chromatin assembly, a drug-induced DNA cleavage assay was employed. Relaxed plasmid DNA was incubated in extracts containing the increasing amount of VM-26. After 3 h incubation, DNA was recovered and analyzed. As shown in Fig. 6 b, in mitotic extract, single-strand cleavage products (nicked circular DNA, form II) and double-strand cleavage products (linear DNA, form III) were observed in a drug dose-dependent manner. In interphase extracts, however, the cleavage reaction was greatly reduced. Judging from the drug concentration that produces the same amount of cleavage products, topo II activity was four- to fivefold higher in mitotic extracts compared with interphase extracts. The same experiments using interphase and mitotic extracts prepared by an alternative method (see Materials and Methods) gave the same results (data not shown). These results suggest that increased catalytic activity of topo II may be important for the assembly of highly condensed chromatin structures in mitotic extracts.
Figure 4. Chromatin condensation in an interphase-to-mitotic assembly pathway. High molecular weight Xenopus genomic DNA was incubated with interphase extracts for 2 h at 20°C (a and b) and then 2 vol of mitotic extract was added to the reaction mixture. After 30 min (c and d), 60 min (e and f), and 120 min (g-j) incubation, assembled structures were fixed and stained with Hoechst 33258 (a, c, e, g, and i) and MPM-2 (b, d, f, h, and j). Bar, 10 μm.
**Effect of a Topo II Inhibitor on Higher-Order Chromatin Assembly**

In a preliminary attempt to answer the question of how topo II functions in higher-order chromatin assembly, a specific topo II inhibitor VM-26 (Chen et al., 1984) was added to chromatin assembly reactions. In interphase extracts, little, if any, effect was observed. We found, however, a striking effect on the chromatin assembly in mitotic extracts. In contrast to rod-shaped structures assembled in the absence of the drug (Fig. 7 a), round-shaped chromatin bodies were formed in the presence of the drug (Fig. 7, c and e). The staining patterns with MPM-2 were also different: in contrast to the punctate or fibrous staining of rod-shaped chromatin structures, round-shaped bodies were uniformly stained. At a low concentration of VM-26 (10 μM), internal dots were still seen in the high background of uniform staining (Fig. 7 d). At a high concentration (40 μM), no internal structures were observed (Fig. 7 f). These results suggest that topo II may play a role in the proper organization of the MPM-2-stained structures and in the final stage of chromatin condensation.

**Discussion**

**Higher-Order Chromatin Assembly in Interphase and Mitotic Extracts**

In this report, we have described an in vitro system in which higher-order chromatin structures are assembled around naked DNA in a cell cycle-dependent manner. One could argue that the "chromatin" structures assembled in our system might be a consequence of random aggregation of DNA molecules in the high concentration of protein present in extracts. Several lines of evidence, however, suggest that the chromatin assembly processes and their products observed in vitro mimic those observed in vivo. First, the chromatin structures assembled in vitro were strictly controlled by the cell cycle states of extracts: interphase extracts promote the assembly of fluffy chromatin-like structures, whereas highly condensed chromosome-like structures were assembled in mitotic extracts. Interphase and mitotic chromatin structures showed the distinct condensation states as judged by their shape and fluorescent intensity. Rough estimation of packing ratio of DNA in highly condensed mitotic chromatin (1,000–5,000) was slightly less but comparable to that of...
metaphase chromosomes (8,000–10,000; Paulson, 1988). These observations were confirmed using interphase and mitotic extracts prepared by a different method, indicating that the higher-order chromatin assembly is truly regulated in the cell cycle. Second, MPM-2, which recognizes a class of mitosis-specific phosphoproteins, stained mitotic chromatin bodies but not interphase chromatin masses, suggesting that the condensation state of chromatin may be directly regulated by mitosis-specific phosphorylation. Consistently, we found that MPM-2 also stains the central axis of mitotic chromosomes isolated from CHO cells. Third, VM-26, a specific topo II inhibitor, blocked the formation of rod-shaped chromatin bodies in mitotic extracts. This is also consistent with the recent in vivo studies that defective condensation of chromatin is observed in the VM-26-treated cells (Charron and Hancock, 1990; Roberge et al., 1990; Wright and Shatten, 1990). Fourth, in the same extracts used for morphological assay, we were able to detect the two defined biochemical activities, nucleosome assembly and cell cycle–dependent topo II activity.

It is of interest to compare our results with those of Newport (1987), who described the pathway of nuclear assembly around naked DNA using interphase crude extracts from Xenopus eggs. In early stages of assembly, we observed similar structural changes of DNA in our interphase soluble extracts. One important difference is that Newport (1987) observed the formation of highly condensed intermediates after the assembly of fluffy chromatin structures, whereas we have observed no indication of further condensation even after longer incubation. The difference cannot simply be explained by the presence of membrane vesicle fractions in his crude extracts because he also observed that in membrane-depleted extracts, DNA transiently formed the condensed intermediates and then decondensed again. On the other hand, our mitotic extracts support completely different structural changes of DNA and the condensed state of the final product is remarkably stable. Although the discrepancy between the two observations in interphase extracts cannot be reconciled at present, it should be emphasized that the transient condensation observed by Newport (1987) in interphase extracts and the stable condensation observed in our mitotic extracts are likely to be regulated by distinct molecular mechanisms.

Cell Cycle Control of Nucleosome Assembly and Topo II Activity
The assembly of higher-order chromatin structures involves successive levels of folding of DNA molecules. The first
level of these folding processes is the assembly of nucleosomes around naked DNA. Although mechanisms of nucleosome assembly have been extensively studied in vitro using Xenopus egg and somatic cell extracts, its regulation in the cell cycle, particularly its dependence on DNA replication, are not fully understood (reviewed by Laskey and Leno, 1990). We compared the kinetics of nucleosome assembly in interphase and mitotic extracts under our standard conditions for chromatin assembly where the nucleosome assembly is uncoupled to DNA replication. We found that nucleosomes for chromatin assembly where the nucleosome assembly in interphase and mitotic extracts under our standard conditions are not fully understood (reviewed by Laskey and Leno, 1990; Adachi et al., 1991). However, mechanisms regulating topo II activity in the cell cycle remain to be investigated, it is plausible to postulate that phosphorylation plays a central role since it has been shown that the activity of purified topo II is increased by phosphorylation in vitro (Ackerman et al., 1985, 1988; Sahyoun et al., 1986; Saijo et al., 1990) and that phosphorylation of topo II reaches a maximum in G2/M phase in vivo (Hirano and Mitchison, 1990). By analogy to many other cell cycle–controlled processes, it is also likely that topo II may be phosphorylated by p34<sup>cdc2</sup> kinase or by downstream kinases of p34<sup>cdc2</sup> (Moreno and Nurse, 1990).

**Assembly of the Chromosome Scaffold-like Structure In Vitro**

It has been shown that a monoclonal antibody MPM-2 recognizes a phosphorylated epitope on a class of proteins that are specifically phosphorylated in mitosis (Davis et al., 1983). One important observation in the present report is that MPM-2 stains the discrete internal structure within mitotic chromatin bodies assembled in vitro. It appears that the assembly of this structure does not require eukaryotic DNA sequences because similar structures were observed in mitotic chromatin bodies assembled around λ DNA. By adding mitotic extracts to interphase assembly mixture (interphase-to-mitotic assembly), we have clearly demonstrated that the highly synchronous condensation of chromatin is accompanied by the structural rearrangements of the MPM-2-stained structures. Furthermore, the addition of VM-26 into mitotic assembly reactions disturbs the organization of these structures and concomitantly affects the final stage of condensation. These results suggest that the assembly of this structure which we term the “condensation core” may play an essential role in chromatin condensation in vitro and that its assembly may be directly regulated by mitosis-specific phosphorylation.

We have also found that MPM-2 stains the central axis of mitotic chromosomes isolated from CHO cells. It is tempting to speculate that the “condensation core” assembled in vitro may be equivalent to this central axis of chromosomes, presumably corresponding to the chromosome scaffold. This notion is supported by our preliminary finding that mitotic chromatin bodies assembled in vitro appear to contain a substructure that is resistant to high salt extraction. The existence of the scaffold structure in intact mitotic chromosomes is still controversial despite the recent accumulation of substantial evidence to support this hypothetical structure (reviewed by Gasser et al., 1989). If the hypothesis that the scaffold is involved in anchoring the chromatin loop domains in both interphase nuclei and mitotic chromosomes is correct, then it seems likely that scaffold proteins are modulated by phosphorylation to rearrange the chromatin loops and subsequently to condense the chromosomes in mitosis.

The molecular nature of MPM-2 antigens localized in the axis of chromosomes and in the condensation core of chromatin bodies assembled in vitro remains to be determined. One possibility is that topo II itself might be one of these antigens. We are currently investigating this possibility.

**A Model for Higher-Order Chromatin Assembly and Condensation In Vitro**

We found that the assembly intermediates observed in “mitotic” and “interphase-to-mitotic” assembly pathways are remarkably different although the final products assembled in the two different pathways have a very similar condensation state. In the direct incubation of DNA with mitotic extracts (“mitotic” assembly pathway), the assembly of the chromosome-like structures seems to be achieved by the mutual association of smaller condensed intermediates, which presumably represents a non-physiological pathway of chroma-
Figure 8. A model for higher-order chromatin assembly and condensation in vitro. In the first step, naked DNA (a, thin line) is assembled into nucleosomes and subsequent levels of folding accompanied by the association of individual DNA molecules result in the formation of fluffy decondensed chromatin (b, thick line). In the second step, putative scaffold phosphoproteins (open circles) bind to the fluffy chromatin, or alternatively interphase chromatin components are modulated by mitosis-specific phosphorylation. Mutual association of these activated proteins induces the formation of partially condensed intermediate (c). In the third step, the putative scaffold components undergo structural rearrangements and produce the central axis of chromatin, leading to the final condensation of mitotic chromatin (d).

In conclusion, we used cell cycle-specific extracts from *Xenopus* eggs to reconstitute higher-order chromatin structures that mimic those observed in vivo. Our in vitro system using naked DNAs as substrates should be useful not only for identifying novel protein components responsible for higher-order chromatin assembly and condensation, but also for testing a possible role of specific DNA sequences implicated in these processes. Furthermore, we are particularly interested in extending this system to the development of a kinetochore assembly system in the future.

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**References**

Ackerman, P., C. V. C. Glover, and N. Osheroff. 1985. Phosphorylation of DNA topoisomerase II by casein kinase II: modulation of eukaryotic topoisomerase II activity in vitro. *Proc. Natl. Acad. Sci. USA.* 82:5164-3168.

Ackerman, P., C. V. C. Glover, and N. Osheroff. 1988. Phosphorylation of DNA topoisomerase II in vivo and in total homogenates of *Drosophila* Kc cells. *J. Biol. Chem.* 263:12653-12660.

Adachi, Y., M. Luke, and U. K. Laemmli. 1991. Chromosome assembly in vitro: topoisomerase II is required for condensation. *Cell.* 64:137-148.

Allan, V. J., and R. D. Vale. 1991. Cell cycle control of microtubule-based membrane transport and tubule formation in vitro. *J. Cell Biol.* 113:347-359.

Amati, B. B., and S. M. Gasser. 1988. Chromosomal ARS and CEN elements bind specifically to the yeast nuclear scaffold. *Cell.* 54:967-978.

Belmont, L. D., A. A. Hyman, K. E. Sawin, and T. J. Mitchison. 1990. Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. *Cell.* 62:579-589.

Benavente, R., G. Krohne, and W. W. Franke. 1985. Cell type-specific expression of nuclear lamina proteins during development of *Xenopus laevis*. *Cell.* 41:177-190.

Blow, J. J., and R. A. Laskey. 1986. Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. *Cell.* 47:577-587.

Blow, J. J., and A. M. Steenman. 1990. Replication of purified DNA in *Xenopus* egg extracts. *Cell.* 47:577-587.

Charron, M., and R. Hancock. 1990. DNA topoisomerase II is required for formation of mitotic chromosomes in Chinese hamster ovary cells: studies using the inhibitor 4'- demethylepipodophyllotoxin 9-(4,6-O-thenylidene-S-0-glucopyranoside).* Biochemistry.* 29:5531-5537.

Chen, G. L., L. Yang, T. C. Rowe, B. D. Halligan, K. M. Tewey, and L. F. Liu. 1984. Nonintercalating antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II.* J. Biol. Chem.* 259:13560-13566.

Cockerill, P. N., and W. T. Garrard. 1986. Chromosomal loop anchorage of the *kappa* immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites.* Cell.* 44:273-282.

Davis, P. M., T. Y. Tsao, S. K. Fowler, and P. N. Rao. 1983. Monoclonal antibodies to mitotic cells.* Proc. Natl. Acad. Sci. USA.* 80:2926-2930.

Dilworth, S. M., S. J. Black, and R. A. Laskey. 1987. Two complexes that contain histones are required for nucleosome assembly in vitro: role of nucleoplasmin and Nl in *Xenopus* egg extracts.* Cell.* 51:1009-1018.

Earnshaw, W. C., B. Halligan, C. A. Cooke, M. M. S. Heck, and L. F. Liu. 1990. Cockerill, P. N., and W. T. Garrard. 1986. Chromosomal loop anchorage of the *kappa* immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites.* Cell.* 44:273-282.

Charron, M., and R. Hancock. 1990. DNA topoisomerase II is required for formation of mitotic chromosomes in Chinese hamster ovary cells: studies using the inhibitor 4'- demethylepipodophyllotoxin 9-(4,6-O-thenylidene-S-0-glucopyranoside).* Biochemistry.* 29:5531-5537.

Chen, G. L., L. Yang, T. C. Rowe, B. D. Halligan, K. M. Tewey, and L. F. Liu. 1984. Nonintercalating antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II.* J. Biol. Chem.* 259:13560-13566.

Cockerill, P. N., and W. T. Garrard. 1986. Chromosomal loop anchorage of the *kappa* immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites.* Cell.* 44:273-282.

Davis, P. M., T. Y. Tsao, S. K. Fowler, and P. N. Rao. 1983. Monoclonal antibodies to mitotic cells.* Proc. Natl. Acad. Sci. USA.* 80:2926-2930.

Dilworth, S. M., S. J. Black, and R. A. Laskey. 1987. Two complexes that contain histones are required for nucleosome assembly in vitro: role of nucleoplasmin and Nl in *Xenopus* egg extracts.* Cell.* 51:1009-1018.

Earnshaw, W. C., B. Halligan, C. A. Cooke, M. M. S. Heck, and L. F. Liu. 1990. Cockerill, P. N., and W. T. Garrard. 1986. Chromosomal loop anchorage of the *kappa* immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites.* Cell.* 44:273-282.

Charron, M., and R. Hancock. 1990. DNA topoisomerase II is required for formation of mitotic chromosomes in Chinese hamster ovary cells: studies using the inhibitor 4'- demethylepipodophyllotoxin 9-(4,6-O-thenylidene-S-0-glucopyranoside).* Biochemistry.* 29:5531-5537.

Chen, G. L., L. Yang, T. C. Rowe, B. D. Halligan, K. M. Tewey, and L. F. Liu. 1984. Nonintercalating antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II.* J. Biol. Chem.* 259:13560-13566.
1985. Topoisomerase II is a structural component of mitotic chromosome scaffolds. J. Cell Biol. 100:1706-1715.

Estey, E., R. C. Adakha, W. N. Hittelman, and L. A. Zweilinger. 1987. Cell cycle stage dependent variations in drug-induced topoisomerase II mediated DNA cleavage and cytotoxicity. Biochemistry. 26:4338-4344.

Gasser, S. M., T. Laroche, J. Falquet, E. Boy de la Tour, and U. K. Laemmli. 1988. Metaphase chromosome structure: involvement of topoisomerase II. J. Mol. Biol. 188:613-629.

Gasser, S. M., B. B. Amati, M. E. Cardenas, and J. F.-X. Hofmann. 1989. The role of cyclin synthesis drives the early embryonic cell cycle. Nature (Lond.). 339:275-280.

Gasser, S. M., T. Laroche, J. Falquet, E. Boy de la Tour, and U. K. Laemmli. 1989. The role of cyclin synthesis drives the early embryonic cell cycle. Nature (Lond.). 339:275-280.

Hirano, T., Y. Hirnoka, and M. Yanagida. 1988. A temperature-sensitive mutation of the Schizosaccharomyces pombe gene nuc2+ that encodes a nuclear scaffold-like protein blocks spindle elongation in mitotic anaphase. J. Cell Biol. 106:1171-1183.

Hirano, T., N. Kinosita, K. Morikawa, and M. Yanagida. 1990. Snap helix with knob and hole: essential repeats in S. pombe nuclear protein nuc2+. Cell. 60:319-328.

Hofmann, J. F.-X., T. Laroche, A. H. Brand, and S. M. Gasser. 1989. RAP-1 factor is necessary for DNA loop formation in vitro at the silent mating type locus HML. Cell. 57:725-737.

Kayne, P. S., K. Ung-Jin, M. Han, R. R. Mullen, F. Yoshizaki, and M. Grunstein. 1988. Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. Cell. 55:27-39.

Laskey, R. A., and G. H. Leno. 1990. Assembly of the cell nucleus. Trends Genet. 6:406-409.

Laskey, R. A., A. D. Mills, and N. R. Morris. 1977. Assembly of SV40 chromatin in a cell-free system from Xenopus eggs. Cell. 10:237-243.

Lohka, M. J., and J. L. Maller. 1985. Induction of nuclear envelope breakdown, chromosome condensation, and spindle formation in cell-free extracts. J. Cell Biol. 101:518-523.

Matthews, H., and E. Bradbury. 1978. The role of histone H1 phosphorylation in the cell cycle: turbidity studies of H1-DNA interactions. Exp. Cell Res. 111:343-351.

Minkovitch, J., M. E. Mirnult, and U. K. Laemmli. 1984. Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold. Cell. 39:223-232.

Mitchison, T. J., and M. W. Kirschner. 1985. Properties of the kinetochore in vitro. I. Microtubule nucleation and tubulin binding. J. Cell Biol. 101:755-765.

Moreno, S., and P. Nurse. 1990. Substrates for p34\textsuperscript{cdk2} in vivo veritas? Cell. 61:549-551.

Murray, A. W., and M. W. Kirschner. 1989. Cyclin synthesis drives the early embryonic cell cycle. Nature (Lond.). 339:275-280.

Murray, A. W., M. J. Solomon, and M. W. Kirschner. 1989. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature (Lond.). 339:280-286.

Newport, J. 1987. Nuclear reconstitution in vitro: stages of assembly around protein-free DNA. Cell. 48:205-217.

Newport, J., and T. Spann. 1987. Dissassembly of the nucleus in mitotic extracts: membrane vesiculation, lamin disassembly, and chromosome condensation are independent processes. Cell. 48:219-230.

Paulson, J. R. 1988. Scaffolding and radial loops: the structural organization of metaphase chromosomes. In Chromosomes and Chromatin. K. W. Adolph, editor. CRC Press, Inc., Boca Raton, FL. 3:3-36.

Paulson, J. R., and U. K. Laemmli. 1977. The structure of histone-depleted metaphase chromosomes. Cell. 12:817-828.

Roberge, M., J. T'ng, J. Hamaguchi, and E. M. Bradbury. 1990. The topoisomerase II inhibitor VM-26 induces marked changes in histone H1 kinase activity, histones H1 and H3 phosphorylation, and chromosome condensation in G2 phase and mitotic BHK cells. J. Cell Biol. 111:1753-1762.

Sahyoun, N., M. Wolf, J. Besterman, T.-S. Hsieh, M. Sander, H. LeVine, K.-J. Chang, and P. C unstreasca. 1986. Protein kinase C phosphorylates topoisomerase II: topoisomerase activation and its possible role in phorbol ester-induced differentiation of HL-60 cells. Proc. Natl. Acad. Sci. USA. 83:1603-1607.

Saujo, M., T. Enomoto, F. Hanoka, and M. U. 1990. Purification and characterization of type II DNA topoisomerase from mouse FM3A cells: phosphorylation of topoisomerase II and modification of its activity. Biochemistry. 29:833-890.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 845 pp.

Sawin, K. E., and T. J. Mitchison. 1991a. Mitotic spindle assembly by two different pathways in vitro. J. Cell Biol. 112:925-940.

Sawin, K. E., and T. J. Mitchison. 1991b. Poleward microtubule flux in mitotic spindles assembled in vitro. J. Cell Biol. 112:941-954.

Thoma, F., T. Koller, and A. Klig. 1979. Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructure of chromatin. J. Cell Biol. 83:403-427.

Uemura, T., H. Ohkura, Y. Adachi, K. Morino, K. Shizoe, and M. Yanagida. 1987. DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in S. pombe. Cell. 50:917-925.

Vale, R. D. 1991. Severing of stable microtubules by a mitotically activated protein in Xenopus egg extracts. Cell. 64:827-839.

Vandere, D. D., F. M. Davis, P. N. Rao, and G. G. Boris. 1984. Phosphoproteins are components of mitotic microtubule organizing centers. Proc. Natl. Acad. Sci. USA. 81:4439-4443.

Vandere, D. D., F. M. Davis, P. N. Rao, and G. G. Boris. 1986. Distribution of cytoskeletal proteins sharing a conserved phosphorylated epitope. Eur. J. Cell Biol. 41:72-81.

Verde, F., J. Labbd, M. Dor6e, and E. Karsenti. 1990. Regulation of microtubule dynamics by cyclin-dependent protein kinase in cell-free extracts of Xenopus eggs. Nature (Lond.). 343:233-238.

Wood, E. R., and C. Earnshaw. 1990. Mitotic chromatin condensation in vitro using somatic cell extracts and nuclei with variable levels of endogenous topoisomerase II. J. Cell Biol. 111:2839-2850.

Wright, S. J., and G. Shatten. 1990. Teniposide, a topoisomerase II inhibitor, prevents chromosome condensation and separation but not decondensation in fertilized surf clam (Spisula solidissima) oocytes. Dev. Biol. 142:224-232.

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