# Mitotic Chromatin Condensation In Vitro Using Somatic Cell Extracts and Nuclei with Variable Levels of Endogenous Topoisomerase II

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*Abstract.* We report the development of a new method for producing mitotic extracts from tissue culture cells. These extracts reproducibly promote the condensation of chromatin in vitro when incubated with purified interphase nuclei. This condensation reaction is not species specific, since nuclei from chicken, human, and hamster cell lines all undergo chromatin condensation upon incubation with the extract. We have used this extract to investigate the role of DNA topoisomerase II (topo II) in the chromosome condensation process. Chromatin condensation does not require the presence of soluble topo II in the mitotic extract. However, the extent of formation of discrete chromosome-like structures correlates with the level of endogenous topo II present in the interphase nuclei. Our results further suggest that chromatin condensation in this extract may involve two processes: chromatin compaction and resolution into discrete chromosomes.

THE higher order structure of chromatin changes dramatically during the growth and division cycle of a eukaryotic cell. As the cell proceeds into mitosis, the interphase chromatin condenses until the metaphase chromosomes visible by light microscopy are formed. The successful condensation of the chromatin is essential to ensure the accurate partitioning of the genome between the daughter cells upon completion of mitosis.

Chromosome condensation involves successive orders of folding of the DNA molecule. Although the exact nature of this folding remains the subject of some controversy, a variety of evidence suggests that chromatin is organized into loop domains of 50–100 kb in both interphase nuclei and mitotic chromosomes (reviewed in reference 12). Because the distribution of histones throughout the chromosome is relatively even, it has been proposed that the loop domains are anchored at their bases by nonhistone proteins. The chromosome scaffold fraction, which consists of a subset of chromosomal proteins that are resistant to solubilization after histone extraction and DNAse treatment, contains proteins that may be involved in anchoring these chromatin loops (1, 6, 24). If the suggestions that scaffold proteins serve a structural role in anchoring chromatin loop domains prove to be correct, then it seems likely that proteins present in this fraction may be involved in chromosome condensation.

The most abundant chromosome scaffold protein is DNA topoisomerase II (topo II)[1] (9, 11). Light and electron microscopy studies of the distribution of topo II in mitotic chromosomes suggest that the enzyme is located at the base of the radial loop domains in native chromosomes (7) and in nuclei (2). This localization pattern is consistent with the known role of topo II in chromosome disjunction at anaphase (4, 21, 40).

It has also been proposed that topo II is involved in chromosome condensation. When *Schizosaccharomyces pombe* is arrested in mitosis through the use of either tubulin mutants or antitubulin drugs, the cells exhibit three highly condensed chromosomes (41). Examination of cold-sensitive (cs) topo II/cs β-tubulin double mutants of *S. pombe* arrested in mitosis at nonpermissive temperature reveals the presence of much elongated prophase-like chromosomes (39). This has been interpreted as evidence for a role of topo II in the final stages of condensation. However, since condensed chromosomes are not normally formed in *S. pombe*, it remains possible that this result is peculiar to this combination of mutations and drug treatments.

A requirement for topo II in chromosome condensation has also been suggested by in vitro experiments using extracts from *Xenopus* eggs. Condensation in these extracts was blocked by the topoisomerase II inhibitors novobiocin and VM26 (30). However, it was unclear if the block in chromosome condensation was due to a specific inhibition of topo II or a nonspecific effect of protein-linked DNA gaps resulting from inhibitor treatment.

We have developed a new in vitro assay for chromatin condensation based upon extracts from mitotic tissue culture cells to investigate the role of chromosome scaffold proteins in the condensation process. Similar previously described mitotic extracts derived from tissue culture cells have proven

1. *Abbreviation used in this paper*: topo II, topoisomerase II.

to be useful for the analysis of nuclear envelope breakdown and lamina solubilization, but failed to yield discrete condensed regions of chromatin as occurs during chromosome formation in vivo (20, 29, 35).

The first scaffold protein we have investigated using this system is DNA topoisomerase II. Our experimental strategy to analyze the role of topo II in condensation relies upon the use of cells of a single developmental lineage as a source of nuclei containing variable levels of topo II. In the erythroid lineage of the embryonic chick, the mitotic index in the peripheral blood falls effectively to zero between 6 and 10 d of development as erythropoiesis becomes localized to specific hematopoietic compartments (31). Earlier studies from this laboratory revealed that levels of topo II observed in peripheral blood cells drop precipitously over this time period as the dividing blast cells are replaced by quiescent primary erythrocytes (16). The loss of topo II from these cells is probably a consequence of normal cell cycle processes, in which the protein accumulates to peak levels during mitosis and is then rapidly degraded during the transition to subsequent G1 (17). Although it is possible that other chromosomal proteins decrease in parallel with topo II over this period of development, we have been unable to detect such a dramatic decrease for any of the other chromosome scaffold proteins for which we have specific antibodies.

In the work reported here, we present the basic characterization of our novel somatic mitotic extract system, together with an assessment of the role of endogenous nuclear topo II levels in chromatin condensation. Using nuclei purified from developing chick erythroblasts as substrates of the reaction, we have shown that the extent of the formation of discrete chromosome-like structures correlates with the level of endogenous topo II present in the added nuclei. Interestingly, overall compaction of the nuclear chromatin does not appear to require topo II. The volume of nuclei containing low levels of topo II decreases by a factor of 2.7 after incubation with extract, but there is no sign of resolution into discretely condensed regions. These results suggest that chromatin condensation in this in vitro system may involve two processes: topo II-independent compaction of the chromatin, and resolution of the condensed chromatin into discrete regions. The latter process may require the presence of endogenous nuclear topo II.

## Materials and Methods

### Cell Culture and Synchrony

Chicken DU249 (hepatoma cells; 22), CHO, and HeLa cells were grown in RPMI 1640 (Gibco Laboratories, Grand Island, NY) plus 5% FBS (HyClone Laboratories, Logan, UT). MSB-1 cells, the gift of H. Weintraub (Hutchinson Cancer Center, Seattle, WA), were grown in RPMI-1640 supplemented with 5% newborn calf serum (HyClone Laboratories). For cell cycle synchronization, DU249 cells were grown in monolayer culture in 150-$cm^2$ tissue culture dishes (Corning Instruments, Corning, NY). When the cultures were ~80% confluent, aphidicolin (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 2 μg/ml. After 12 h of incubation, cells were released into fresh culture medium. Cell culture was continued for 7 h, at which point nocadozole (Sigma Chemical Co.) was added to a final concentration of 50 ng/ml. After 3 h, ~75% of the cells were arrested in metaphase, and were recovered by mechanical shakeoff (37). Using this procedure, 90–95% of the cells obtained were in mitosis, as indicated by the lack of a nuclear membrane and the presence of condensed mitotic chromosomes.

Chicken erythrocytes from various stages of embryonic development were obtained from chicken embryos (Truslow Farms, Chestertown, MD) as previously described (16).

### Preparation of Mitotic Extracts

For a typical extract production, 10–15 150-$cm^2$ flasks of DU249 cells were synchronized; the cells were collected as described above. All steps subsequent to cell harvest were performed on ice. Mitotic cells were washed in 20 vol of ice-cold incomplete lysis buffer (50 mM Pipes-KOH, pH 7.0, 50 mM KCl, 10 mM EGTA, 2 mM $MgCl_2$, adapted from reference 35), followed by a subsequent wash in 20 vol of complete lysis buffer (lysis buffer plus 1 mM DTT, 2 μM cytochalasin B [Sigma Chemical Co.], 0.1 mM PMSF, 1 μg/ml CLAP [chymostatin, leupeptin, antipain, and pepstatin A]). The cells were then resuspended in 1 vol of complete lysis buffer, and pelleted (325 *g*, 2 min) directly into two to four 0.5-ml glass microtissue homogenizers (Wheaton Instruments Div., Milville, NJ). The supernatant was removed, and the pelleted cells were quick frozen in a −80°C 80% isopropanol slush. The frozen cells were lysed by grinding as the pellet thawed. This freeze–thaw lysis procedure was repeated twice. A crude cytoplasmic fraction was obtained from the lysed cells by low-speed centrifugation (27,000 *g* for 15 min). The supernatant was collected and clarified by high-speed centrifugation (150,000 *g* for 3 h). This soluble mitotic extract (supernatant) was then divided into 80-μl aliquots and stored under liquid nitrogen where it remained active for at least 2 mo. Using this procedure, the mitotic extract typically has a protein concentration of 50–60 mg/ml.

Interphase extracts were prepared from logarithmically growing DU249 cells harvested with a rubber policeman. All subsequent steps were identical to those described above.

### Purification of Interphase Nuclei

For isolation of interphase nuclei, 10 μM cytochalasin B was added to logarithmically growing MSB-1 cells and incubation was continued for 30 min. The cells were then harvested by centrifugation and washed in 20 vol of PBS (Dulbecco's formulation plus 1 mM $MgCl_2$). A second wash was performed in 20 vol of NB (10 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 10 μM cytochalasin B, 0.1 mM PMSF, 1 μg/ml CLAP). The washed cells were resuspended in 2 vol of NB and allowed to swell for 20 min on ice. After swelling, the cells were gently lysed by 20 strokes in a 15-ml Dounce homogenizer. Up to 2 ml of homogenate were layered over 4.5 ml of NB plus 30% sucrose in a 40-ml round-bottom centrifuge tube, and the nuclei were pelleted by centrifugation at 500 *g* for 3 min. The nuclei were then washed in 1 ml of complete lysis buffer and resuspended at a final concentration of $2 \times 10^8$/ml.

Nuclei from developing chick erythroblasts were isolated as described above except cell lysis was accomplished with more vigorous and more numerous (50–100) strokes of the Dounce homogenizer.

### In Vitro Chromatin Condensation Reactions

Typical in vitro chromatin condensation reactions (20 μl) contained, 15 μl of mitotic extract ($1 \times 10^7$ cell equivalents), 3 μl of purified nuclei ($1.5 \times 10^6$), and an ATP-regenerating system consisting of 2 mM ATP, 10 mM creatine phosphate, and 50 μg/ml creatine kinase. Incubations were performed in a 0.5-ml microfuge tube at 37°C for 90 min. For buffer controls, the mitotic extract was replaced with 15 μl of lysis buffer.

To observe chromatin condensation, 10-μl aliquots were withdrawn and fixed by adding 2 vol of 4% formaldehyde in PBS for 5 min. The fixed nuclei were then gently centrifuged onto coverslips, stained with the DNA-specific dye, diaminophenylindole (DAPI), and visualized by fluorescence microscopy.

Determination of nuclear volume before and after incubation with the mitotic extract was performed using a Hypervision digital image-processing system (Perceptics, Knoxville TN). Briefly, the system defined the borders of objects in an image above a defined threshold of light intensity. The computer then calculated the area in pixels for each of the identified objects. Volumes for the nuclei were calculated using the value of the radius of each nucleus and assuming that the nuclei were spheres. For noncondensed nuclei and nuclei incapable of forming discrete condensed regions (late-stage erythrocyte nuclei) the volume represents the region occupied by the DAPI-stained chromatin. For nuclei condensed into chromosome-like structures the volume represents the entire region encompassed by the condensed material (i.e., the condensed chromosome-like structures and the area in between them).

### *Immunofluorescence*

For lamin immunofluorescence experiments, condensation reactions were performed as described above, only the reactions were carried out using nuclei that were preattached to adhesioslides (MM Developments, Ottawa, Canada). After a standard incubation with buffer or mitotic extract, the nuclei were fixed and processed for indirect immunofluorescence as described previously (10). The anti-chicken lamin B2 antibody (23) was the generous gift of Dr. Erich Nigg (ISREC, Lausanne, Switzerland).

Quantitation of the intensity of lamin immunofluorescence was performed using the Hypervision system. Images of nuclei treated with buffer or extract and stained with antilamin antibodies were acquired at identical gain and contrast settings of the SIT camera (DAGE-MTI Corp., Michigan City, IN). Nuclei were then randomly selected and the average intensity of fluorescence within each nucleus was automatically determined. Fluorescence intensity was expressed as a value between 0 and 1, with 1 being the highest intensity and 0 being completely black. At least 20 individual nuclei were averaged to generate each mean value of fluorescence intensity.

### *Quantitation of Topo II Levels*

Previous work from this laboratory has determined that rapidly growing MSB-1 cells contain $4.9 \times 10^5$ copies of topo II per cell (15), thus topo II levels were estimated for mitotic extracts and chick erythroblast nuclei by comparative immunoblotting using rapidly growing MSB-1 cells as a standard. A range of MSB-1 cells ($5 \times 10^4$–$10^5$) were lysed in SDS-sample buffer and loaded onto a 10% polyacrylamide gel. Variable amounts of mitotic extract or chicken erythroblast nuclei were processed in a similar fashion and loaded onto the same gel. An immunoblot of this gel was subsequently performed as described previously (8), and probed with a specific rabbit anti-topo II antibody (19). Antibody-antigen complexes were detected using iodinated protein A and visualized by autoradiography. After autoradiography the immunoblot was stained with India ink and appropriate regions of the nitrocellulose filter were cut out and counted in a gamma counter. A standard curve of counts per minute vs. topo II copy number was therefore generated using the MSB-1 cells, and the levels of topo II in the mitotic extract and in the purified chick erythroblast nuclei were estimated using this standard curve.

### *Immunodepletions*

Immunodepletions of topo II from the mitotic extracts were performed using topo II-specific guinea pig antibody 2B2 (9). Fixed *Staphylococcus aureus* cells (Pansorben; Pharmacia Fine Chemicals, Piscataway, NJ) were washed three times in 10 vol of PBS plus 4% BSA and resuspended in 1 volume of this solution. 20 μl of guinea pig 2B2 serum or preimmune serum were then incubated with 20 μl of washed Pansorben while rotating on a mechanical shaker at 4°C for 1 h. The antibody-preadsorbed Pansorben was then washed as described above. After the final wash, the Pansorben pellet was resuspended with 20 μl of mitotic extract. The extract and Pansorben were incubated on ice with intermittent mixing for 1 h. The Pansorben was then separated from the extract by centrifugation, and 15 μl of treated extract were removed and used in a chromatin condensation reaction. 2 μl of remaining extract were then analyzed for topo II levels by immunoblotting. The Pansorben pellets were washed three times with 50 vol of KB per wash (150 mM NaCl, 10 mM Tris-HCl [pH 7.7], 0.1% Triton X-100, 0.1% BSA), resuspended in 40 μl of SDS-sample buffer, boiled for 5 min, centrifuged briefly to pellet the Pansorben, and loaded onto the same polyacrylamide gel as the extract supernatants.

## *Results*

### *Mitotic Extracts from Somatic Cells Promote Chromatin Condensation In Vitro when Incubated with Purified Interphase Nuclei*

Highly concentrated (50–60 mg/ml protein) extracts prepared from mitotic cells of the chicken hepatoma-derived line DU249 induce chromatin condensation in added interphase nuclei (Fig. 1, *c* and *d*). In a typical in vitro chromatin condensation reaction, the extract is incubated with purified interphase nuclei from the chicken lymphoblastoid cell line, MSB-1 in the presence of an ATP-regenerating system at 37°C for 90 min. Under these conditions, the chromatin in the extract-treated nuclei is condensed into numerous discrete structures (Fig. 1, *c* and *d*). The observed condensation is not due to any components of the lysis buffer, since cells treated with buffer alone do not exhibit any change in the condensation state (Fig. 1, *a* and *b*).

Condensation of the interphase chromatin is first observed after 20 min incubation with the extract and is complete after 60–90 min. The condensation reaction is also relatively sensitive to dilution of the mitotic extract. Dilution of the extract by a factor of 3 abolishes chromatin condensation (data not shown).

A cytological spread of DAPI-stained mitotic chromosomes from DU249 cells (diploid chromosome number ~60) is shown in Fig. 2 *a*. A comparison of DU249 nuclei (Fig. 2 *b*) or MSB nuclei (Fig. 2 *c*) condensed in vitro reveals that the products of the condensation reaction are roughly similar to naturally occurring chromosomes in both size and number.

### *Chromatin-condensing Activity Is Enhanced in Mitotic Extracts*

We have prepared identical extracts from mitotic cells (M extracts), logarithmically growing cells (log extracts), or cells blocked at the G1/S boundary by aphidicolin treatment (S extracts) to investigate the effect of the cell cycle phase from which the extract is made upon chromatin condensation in vitro. As expected chromatin condensation of added nuclei occurs with high frequency in M extracts (Fig. 3 *b*). The proportion of nuclei condensed using these extracts ranged from 82–98% (50 different M extracts have been prepared). In some of our interphase extracts (four different log extracts and two S extracts have been tested) we observed low and variable levels of apparent chromatin condensation (Fig. 3 *a*, nucleus on top right is slightly condensed). However, dilution of the extracts reveals that the chromatin-condensing activity is consistently much higher in mitotic extracts than in interphase extracts.

### *Basic Characteristics of the In Vitro Chromatin Condensation Reaction*

Chromatin condensation using this somatic system is sensitive to increased concentrations of divalent cations. More than 2 mM $Ca^{2+}$ and >10 mM $Mg^{2+}$ added to the reaction result in essentially complete inhibition of chromatin condensation. The reaction is also sensitive to Tris buffers (>4 mM inhibits condensation) and DMSO (>3% inhibits condensation). Heat treatment of the extract at 65°C for 10 min also abolishes chromatin condensation. Together, these observations suggest that the condensation observed is not due to nonspecific aggregation of the chromatin.

### *Mitotic Extracts Promote Chromatin Condensation in Nuclei from a Variety of Sources*

In addition to nuclei from chicken MSB-1 cells, nuclei from untransformed chicken fibroblasts (not shown), HeLa (Fig. 4 *a*), CHO (Fig. 4 *b*) and even *Drosophila* tissue culture cells (not shown) undergo chromatin condensation when incubated with the extracts. The proportion of these nuclei that

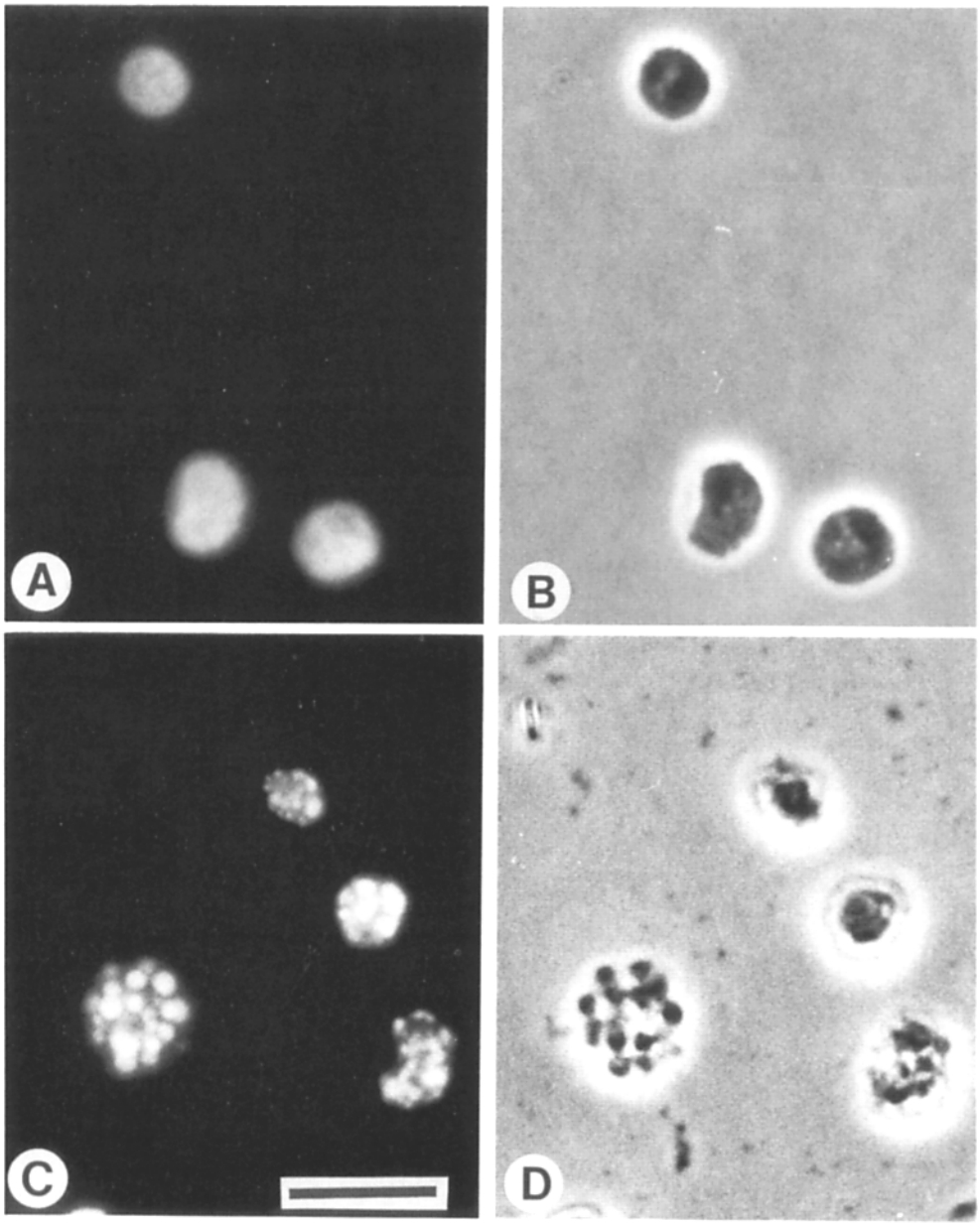


*Figure 1.* Chromatin condensation in vitro using mitotic extracts from somatic cells. Purified MSB-1 nuclei were treated with lysis buffer (*a* and *b*) or mitotic extract (*c* and *d*), fixed with 3% formaldehyde in PBS, and gently centrifuged onto coverslips. (*a* and *c*) DAPI staining. (*b* and *d*) Phase contrast. Bar, 10 $\mu$M.

condenses upon incubation with the extract is essentially identical to the proportion of MSB-1 nuclei that condense (>90%). The number of condensed chromosome-like structures seen when nuclei from these various cell types are used as substrates of the condensation reaction roughly correlates with the chromosome number for each cell type.

### *The Somatic Mitotic Extract Catalyzes the Solubilization of the Nuclear Lamina*

We have investigated nuclear lamina solubilization during the in vitro chromatin condensation reaction by immunofluorescence with an antibody to chicken lamin B2. Purified MSB-1 nuclei treated with buffer alone are stained preferentially around the nuclear periphery by the lamin B2 antibody in a fashion similar to that previously reported for the nuclear lamina (Fig. 5 *b*) (13), and the chromatin remains uncondensed (Fig. 5 *a*). The intensity of lamina staining with the lamin B2 antibody is greatly reduced upon incubation of purified MSB-1 nuclei with the mitotic extract (Fig. 5 *d*) and chromatin condensation occurs as usual (Fig. 5 *c*). The reduction in lamin B2 staining intensity is presumably due to the solubilization of the nuclear lamina.

The degree of lamina solubilization has been estimated by quantitation of the lamin B2 immunofluorescence intensities. The average intensity of lamin B2 immunofluorescence in the buffer treated nuclei was 0.72, (arbitrary units, averaged for 20 randomly picked nuclei) whereas the average intensity of lamin immunofluorescence in the extract treated nuclei was 0.09. Thus, lamin B2 immunofluorescent intensity is reduced by ~87% upon treatment with mitotic extract.

### *Chromatin Condensation In Vitro Using MSB-1 Nuclei Is Not Dependent upon Soluble Topoisomerase II in the Mitotic Extract*

Our motivation for developing the in vitro chromatin con-

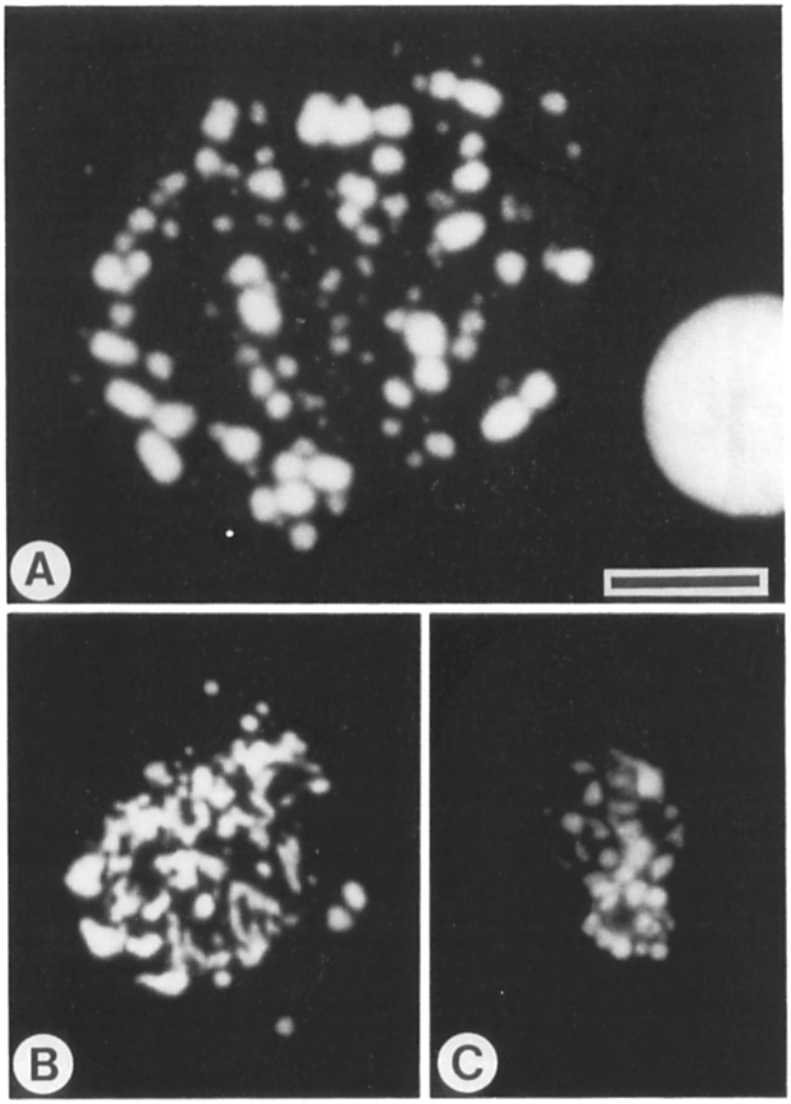


*Figure 2.* The products of the in vitro chromatin condensation reaction compared to naturally formed mitotic chromosomes. (*A*) Cytological spread of mitotic chicken DU249 cell stained with DAPI. (*B* and *C*) Nuclei purified from either chicken DU249 (*B*) or MSB-1 (*C*) cells were treated with mitotic extract and processed as described for Fig. 1. Bar, 10 $\mu$M.

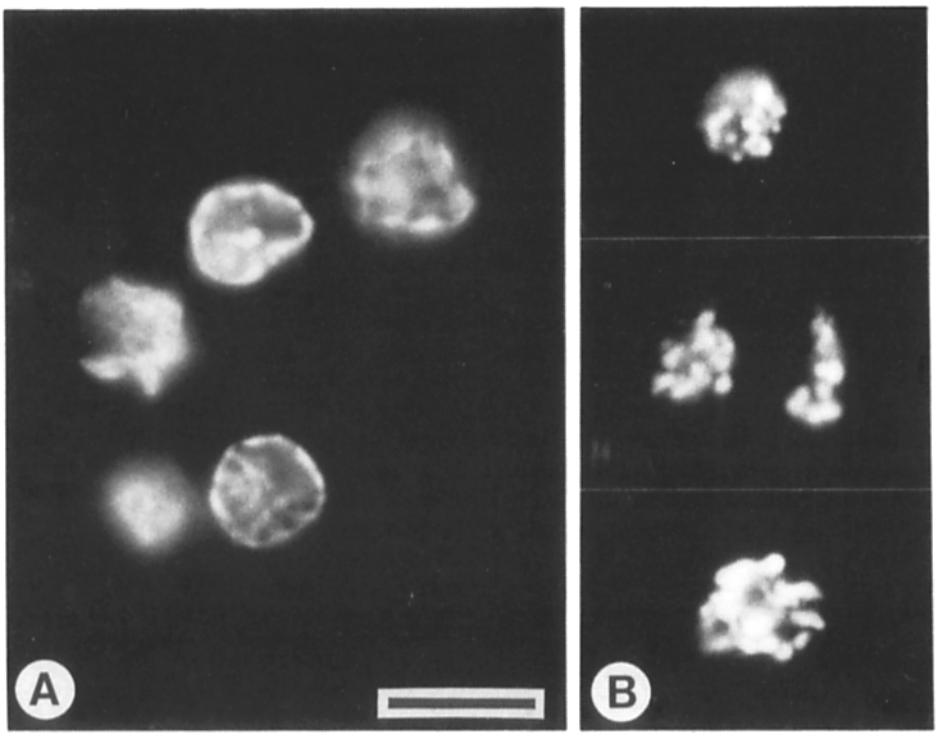


*Figure 3.* Chromatin condensation in vitro using extracts from logarithmically growing or mitotic cells. Identical extracts were prepared from logarithmically growing or mitotic DU249 cells and incubated with purified MSB-1 nuclei. (*A*) MSB-1 nuclei treated with log extract. (*B*) MSB-1 nuclei treated with mitotic extract. Bar, 10 $\mu$M.

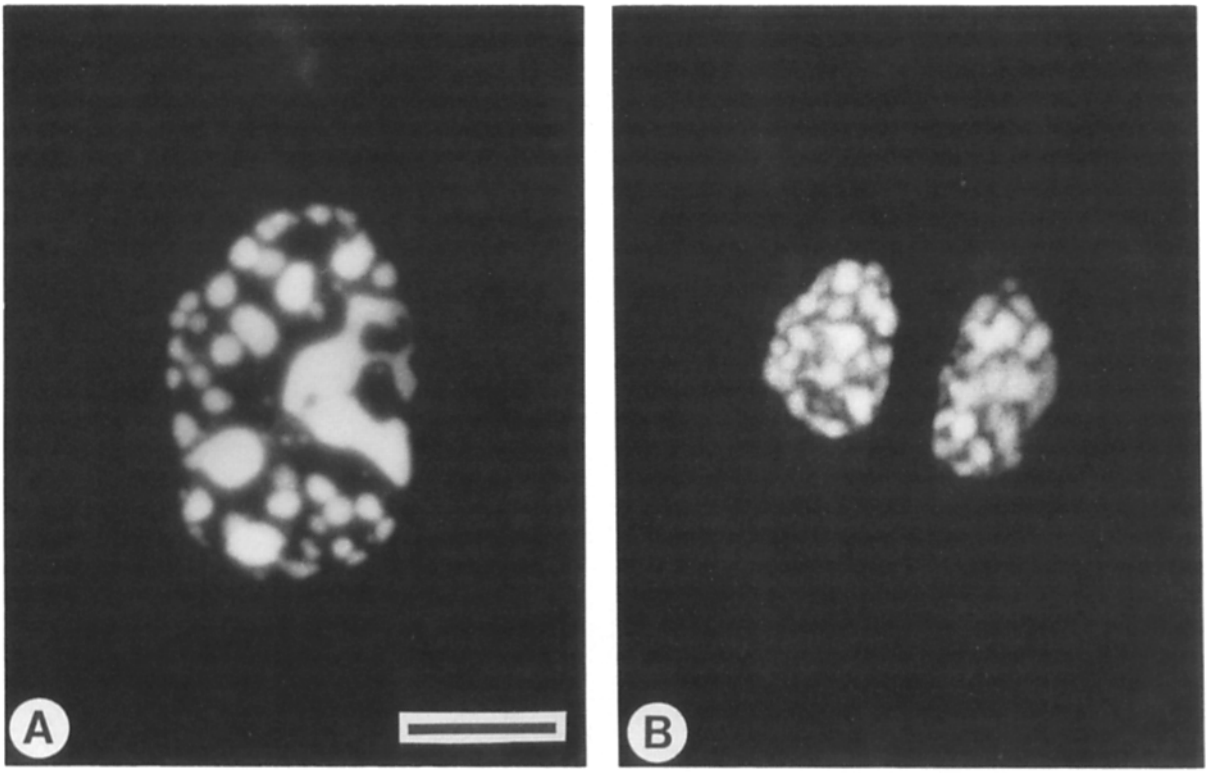


*Figure 4.* Incubation of human and hamster cells with mitotic extract. Nuclei purified from (*A*) HeLa and (*B*) CHO cells were incubated with mitotic extract, stained with DAPI, and processed as described for Fig. 1. Bar, 10 μM.

densation system was to provide a means to investigate the role of individual chromosome scaffold proteins in the chromosome condensation process. Somatic cell extracts may be ideal for such a study since they do not contain large stockpiles of nonhistone chromosomal proteins as do extracts derived from amphibian eggs. The first scaffold protein we have examined using this system is DNA topoisomerase II.

Our strategy for testing the hypothesis that topo II is required for chromosome condensation has been to ask whether condensation can occur in the absence of the protein. This strategy is complicated by the fact that topo II is provided by two sources in a typical in vitro condensation experiment. The enzyme is present in a soluble form in the mitotic extract, and also is tightly coupled to the chromatin in the purified MSB-1 nuclei.

The level of topo II in actively growing chicken DU249 cells (from which the extract is made) has been determined by quantitative immunoprecipitation to be $4.9 \times 10^5$ monomers per nucleus (15). Therefore, the level of topo II in the mitotic extract can be accurately determined by comparative immunoblotting using DU249 cells as a standard. We have used this quantitative immunoblotting approach to determine that our mitotic extracts retain only 2% of the total topo II originally present in the DU249 cells. This very low recovery of soluble topo II is not surprising since the chromosomes of the mitotic DU249 cells are removed from the soluble extract by sedimentation during extract preparation, and it has been previously shown that topo II is tightly associated with mitotic chromosomes (9, 11).

Even though the level of topo II in the mitotic extract is low, it is possible that the soluble topo II is enough to fulfill any requirement for the enzyme in the condensation reaction. We have tested the requirement for soluble topo II by immunodepletion of the enzyme from the mitotic extract using an anti-topo II antibody (9). Antibody treatment quantitatively removes soluble topo II from the extract (Fig. 6). The level of topo II in the untreated extract is shown in Fig. 6, lane *1*. Topo II-specific polyclonal antibodies completely immunoprecipitate all detectable topo II from the mitotic extract (Fig. 6, lane *4*). The precipitated topo II is recovered in the pellet fraction (Fig. 6, lane *8*). The levels of topo II are not significantly affected by treatment with preimmune serum, or formaldehyde fixed *S. aureus* cells alone (Fig. 6, lanes *2* and *3*).

When the topo II-immunodepleted mitotic extract is incubated with MSB-1 nuclei, chromatin condensation occurs in a fashion indistinguishable from the condensation catalyzed by the untreated extract (compare Fig. 7, *b* and *c*). Extracts mock-depleted with either preimmune serum or immunoadsorbant alone also retain full activity (not shown). This indicates that if topo II is required for chromatin condensation, then endogenous enzyme present in the nuclei themselves is enough to fulfill this requirement.

Previous results have shown that topo II is tightly associated with the nuclear matrix in interphase nuclei (2). It is therefore not practical to remove the enzyme from nuclei by immunodepletion as described above. Initial attempts to block chromatin condensation by preincubating the interphase nuclei with various topo II antibodies (two polyclonals and one monoclonal) have been unsuccessful. However, it is possible that epitopes recognized by the antibodies are masked in interphase nuclei, or that the antibodies do not in-

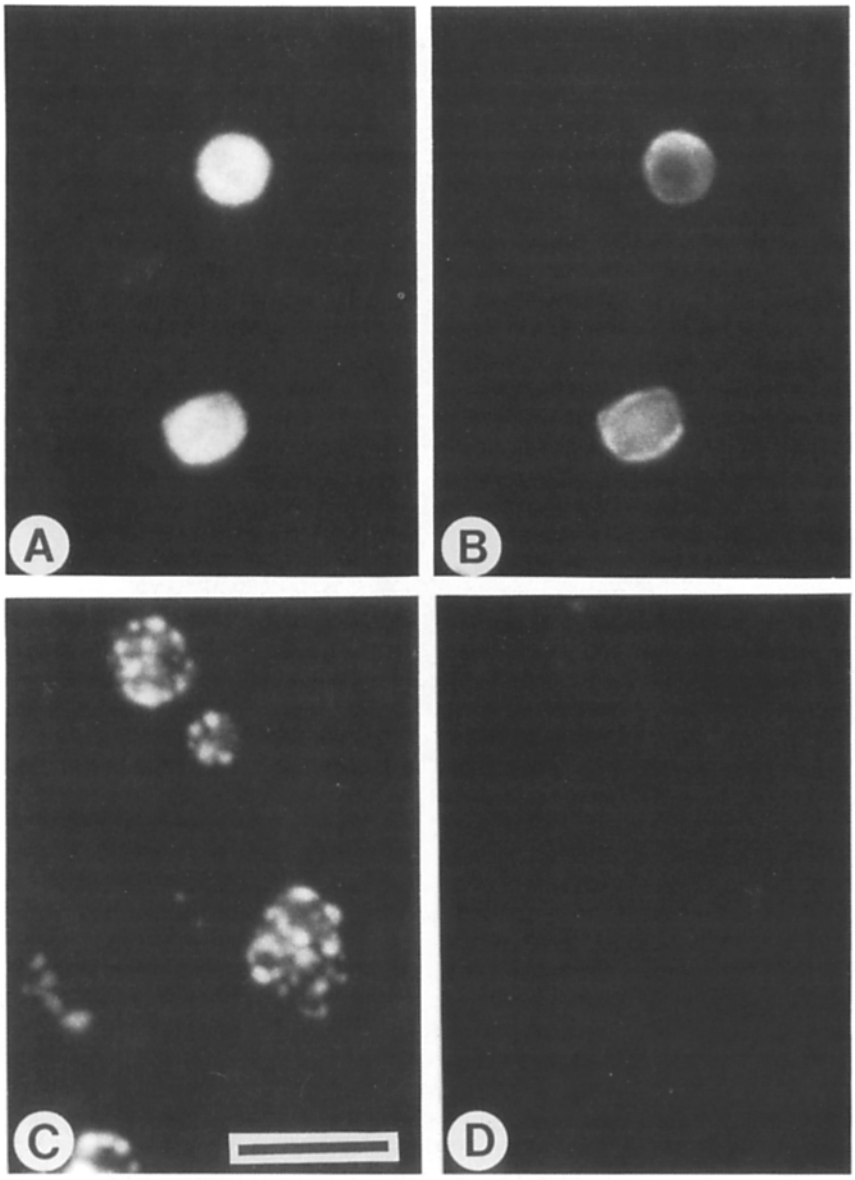


*Figure 5.* Nuclear lamina solubilization accompanies chromatin condensation in vitro using mitotic extracts. Nuclei purified from MSB-1 cells were treated with lysis buffer (*A* and *B*) or mitotic extract (*C* and *D*). (*A* and *C*) DAPI staining of DNA. (*B* and *D*) visualization of the nuclear lamina by immunofluorescence with a chicken lamin B2-specific antibody. Bar, 10 $\mu$M.

terfere with the function of the protein. To examine the role of endogenous nuclear topo II in condensation, we have therefore resorted to the use of a system where nuclei naturally devoid of topo II are used as substrates for the condensation reaction.

### *In Vitro Condensation of Chromatin from Chick Erythroblast Nuclei Varies with the Level of Endogenous Topo II*

Previous work from many laboratories has demonstrated that topo II is expressed preferentially in actively proliferating cells (5, 16, 33, 36). Specifically, we have shown that during chicken embryogenesis, total cellular topoisomerase II levels in erythroblasts decrease dramatically concomitant with the cessation of cell division (16). As shown in Table I, the level of topoisomerase II in MSB-1 cells is $4.9 \times 10^5$ copies per cell. By quantitative immunoblotting, we have determined that there are $1.1 \times 10^5$ copies of topo II per cell in erythroblasts from 3-d chick embryos. This level is relatively stable until day 6 of embryogenesis, when the level begins to rapidly decrease. In erythroblasts from day 7 embryos, there are $4 \times 10^4$ copies per cell, and by day 9 there are only $1.6 \times 10^4$ copies per primitive erythrocyte. In mature chick erythrocytes, topo II is undetectable by immunoblotting (i.e., <100 copies per cell) (15).

We have taken advantage of the erythroid developmental lineage to examine the relationship between the level of endogenous topo II and competence in our chromatin condensation assay. Nuclei from day 3 erythroblasts readily undergo chromatin condensation when incubated with mitotic extract (Fig. 8 *b*). We consistently noted that the degree of condensation is not as complete as when MSB-1 nuclei are used as substrate (Fig. 1 *b*). This may be the result of either the lower levels of endogenous topo II or other changes in nuclear structure.

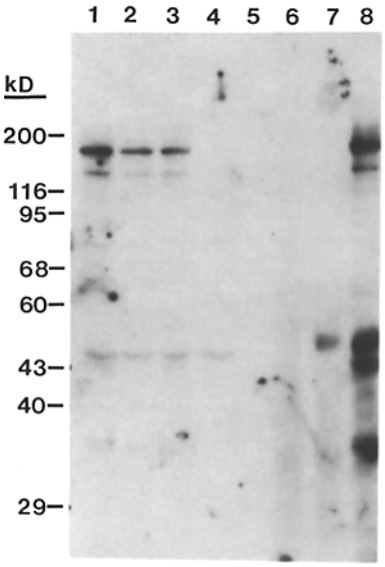


*Figure 6.* Immunodepletion of topoisomerase II from mitotic extract. Lanes *1–4*, 2 μl of mitotic extract after immunodepletion (supernatant) were diluted with sample buffer to 20 μl; and electrophoresed on a 10% SDS polyacrylamide gel. The gel was transferred to nitrocellulose and probed with antitopo II antibody. Lane *1*, untreated mitotic extract. Lane *2*, mitotic extract treated for immunodepletion using Pansorben only. Lane *3*, mitotic extract treated for immunodepletion using preimmune serum and Pansorben. Lane *4*, mitotic extract treated for immunodepletion using anti-topo II antibodies and Pansorben. Lanes *6–8*, the immunoprecipitated material (pellet) was released from the Pansorben by boiling in sample buffer and analyzed by immunoblotting on the same gel. Lanes *6*, pellet from immunodepletion with Pansorben only. Lane *7*, pellet from immunodepletion with preimmune serum and Pansorben. Lane *8*, pellet from immunodepletion with anti-topo II antibodies and Pansorben.

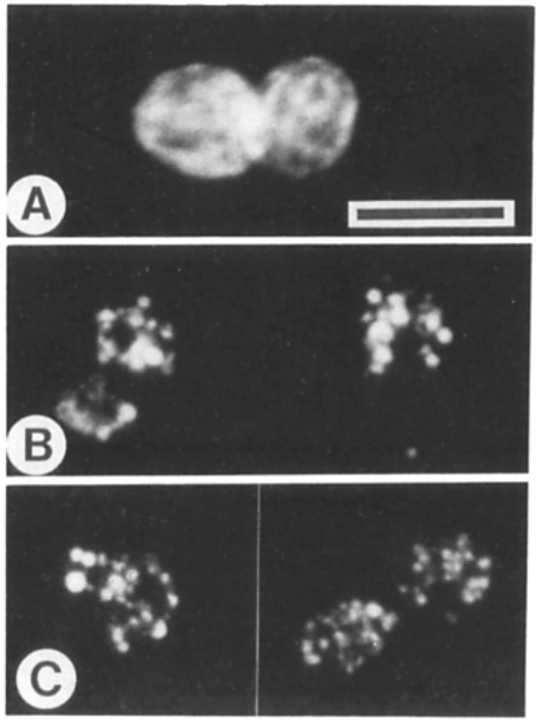


*Figure 7.* Chromatin condensation in vitro using extract lacking detectable topoisomerase II. Nuclei purified from logarithmically growing MSB-1 cells were incubated with (*A*) lysis buffer, (*B*) mitotic extract, and (*C*) mitotic extract from which all detectable topo II has been removed by immunodepletion. Topo II levels present in the extracts used in this experiment are shown in Fig. 6 (lane *1*, mitotic extract, lane *4*, topo II-depleted mitotic extract). Bar, 10 μM.

We have used a counting assay to estimate the ability of nuclei to form discrete chromosome-like structures when incubated with this extract. Briefly, we score a nucleus as condensed if at least four distinct regions of condensed chromatin are visible after incubation with the extract followed by DAPI staining. Nuclei with fewer than four distinct condensed regions are scored as not condensed. Using this method, we determined that 56% of the 3-d erythroblast nuclei condense under these conditions (Table I). Longer incubations do not change the extent of condensation or the percentage of nuclei condensed. Using MSB-1 nuclei as substrate, virtually 100% of the nuclei condensed in this extract.

Nuclei from 5-d erythroblasts also condense when incubated with mitotic extract (Fig. 8 *d*). The proportion of nuclei condensed using 5-d erythroblasts decreased to 36% (Table I). Using 7-d erythroblasts, only 29% of the nuclei are condensed, and the majority of the nuclei appear to be reduced in volume (Fig. 8 *f*). Nuclei from 9-d chick primitive erythrocytes are unable to condense into discrete structures when incubated with mitotic extracts (Fig. 8 *h*).

Interestingly, measurements of the area of the DAPI-stained chromatin indicate that the volume occupied by the chromatin within the 7- and 9-d nuclei decreases upon exposure to mitotic extract (Table II). Areas of images of the nuclei used in this study were determined using the Hypervision image processing system, and the corresponding nuclear volumes were determined (see Materials and Methods). The total volume occupied by the condensed chromosome-like structures (condensed structures and area in between them) in MSB, 3-d, and 5-d nuclei is roughly equivalent to the original nuclear volume (Table II). However, the volume occupied by the chromatin in the 7- and 9-d nuclei is reduced by more than twofold upon treatment with mitotic extract. Thus, even though the chromatin within the 7- and 9-d nuclei does not resolve into discrete structures, it is compacted into a smaller volume.

*Table I. Endogenous Nuclear Topo II Levels Correlate with Condensation Ability*

| Nuclei | No. of topo II monomers (× $10^5$/nucleus) | Condensation |
|---|---|---|
| | | % |
| MSB-1 | 4.9 | >90 |
| 3-d erythroblast | 1.1 | 56 |
| 5-d erythroblast | 0.99 | 36 |
| 7-d erythroblast | 0.40 | 29 |
| 9-d erythrocyte | 0.16 | <5 |

The number of topo II monomers per nucleus was estimated as described in Materials and Methods. The percent condensation represents the percentage of nuclei containing at least four discretely condensed regions.

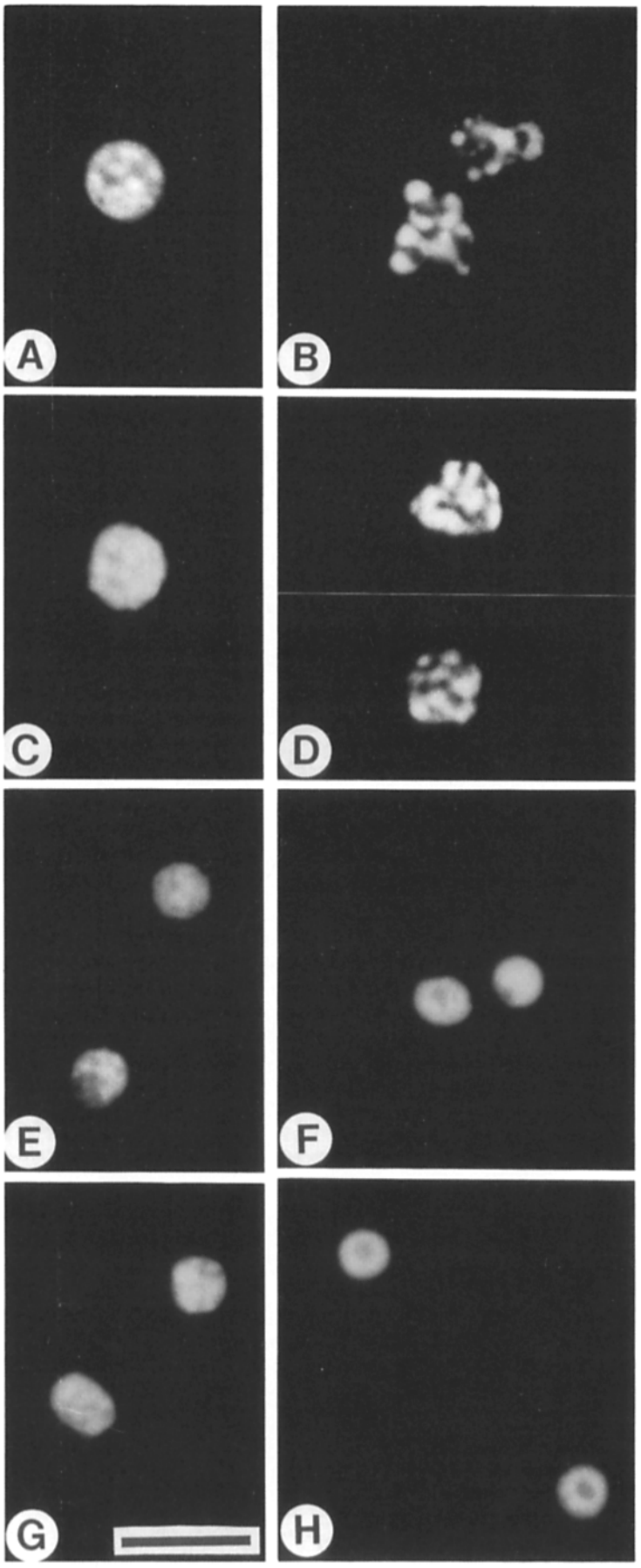


*Figure 8.* Chromatin condensation using nuclei containing variable levels of endogenous topoisomerase II. Purified erythroblast nuclei from various stages of chicken development were incubated with lysis buffer (*A*, *C*, *E*, and *G*) or mitotic extract (*B*, *D*, *F*, and *H*). (*A* and *B*) 3-d erythroblast nuclei. (*C* and *D*) 5-d erythroblast nuclei. (*E* and *F*) 7-d erythroblast nuclei. (*G* and *H*) 9-d primitive erythrocyte nuclei. Bar, 10 $\mu$M.

*Table II. The Effect of Treatment with Mitotic Extract on Nuclear Volume*

| Nuclei | Volume buffer-treated | Volume extract-treated | Original volume |
|---|---|---|---|
| | $\mu M^3$ | | % |
| MSB-1 | 785 ± 123 | 1,119 ± 211 | 142 |
| 3-d | 497 ± 26 | 584 ± 93 | 118 |
| 5-d | 326 ± 40 | 227 ± 34 | 70 |
| 7-d | 429 ± 7 | 187 ± 23 | 44 |
| 9-d | 316 ± 33 | 117 ± 11 | 37 |

Nuclear volumes were calculated as described in Materials and Methods. Each volume represents the average of 20 individual nuclei. For condensed nuclei the volume was calculated based upon the area contained within the complete residual nucleus.

These results demonstrate that the degree of resolution of interphase chromatin into discrete condensed regions correlates with the level of endogenous topoisomerase II, but that the compaction of the chromatin does not require this enzyme.

## Discussion

Although much progress has been made recently in understanding events that control the entry into mitosis, very little is known about the immediate events leading to mitotic chromosome condensation. To further our understanding of the details of this process, we have developed a somatic cell extract that promotes chromatin condensation of added interphase nuclei in vitro.

Previously described in vitro chromosome condensation systems developed from *Xenopus* egg extracts yield chromosomes with excellent morphology (26, 28, 30). However, these extracts contain large stockpiles of chromosomal proteins ($\sim 1.4 \times 10^{10}$ monomers of topo II per egg, for example [27]), and apparently use these to remodel substrate nuclei before chromosome condensation (14, 28). For this reason, *Xenopus* extracts may not be ideal for experiments designed to take advantage of the particular protein content of the substrate nuclei. Thus we have developed tissue culture cell extracts that do not contain large stockpiles of chromosomal proteins to drive chromosome condensation. These extracts also enable us to take advantage of specific antibody probes to chromosome scaffold proteins developed previously in this laboratory (3, 9, 17, 19, 32).

Using our somatic extract system, we hope to identify specific structural proteins or modifications to such proteins directly involved in the condensation process. For the results of such an approach to be valid, it is of utmost importance that the reaction observed in vitro be an accurate model of what occurs in vivo. Several lines of evidence suggest that our in vitro system meets this criterion.

(*a*) The in vitro chromatin condensation reaction results in the formation of discrete condensed regions of chromatin. These regions are frequently linear, like mitotic chromosomes, and the number of structures observed roughly correlates with the karyotype of the substrate nuclei (Fig. 2). Although the structures formed in vitro are similar to the early condensation foci observed in the elegant in vivo analysis of chromosome condensation in *Drosophila melanogaster* embryos (18), it will be necessary in future experiments to follow the in vitro condensation process in real time to assess the extent to which the in vivo and in vitro processes resemble on another. At present we cannot determine if mitotic chromosomes per se are formed during the reaction, or if the structures observed represent some intermediate stage of the condensation process.

(*b*) Nuclear lamina solubilization, which also occurs during normal mitosis, accompanies chromatin condensation in vitro (Fig. 5). An interesting aspect of this reaction is that the condensed regions of chromatin often remain associated even though the nuclear lamina is ~87% solubilized. This continued association may be mediated by a low level of unsolubilized nuclear lamins, residual nuclear membrane, or incomplete untangling of intertwined DNA strands during the condensation process.

(*c*) The in vitro chromatin condensation reaction is inhibited by several factors that, if anything, might be expected to increase the degree of nonspecific chromatin aggregation, such as increased concentrations of divalent cations. Brief heat treatment of the extract also abolishes condensation, implying that the reaction requires extract proteins in their native state.

(*d*) Chromatin-condensing activity is greatly enhanced in extracts from mitotic cells (Fig. 3). Mitotic extracts contain consistently high levels of condensing activity (80–100% of nuclei condense). We have observed low and variable levels of chromatin condensation in extracts prepared from logarithmically growing cells, but the level of condensing activity in these extracts is much lower than that observed with mitotic extracts. The reason for the low level of condensing activity in interphase extracts is not known, but may be the result of the artifactual activation of $p34^{cdc2}$ by phosphatases liberated during the freeze–thaw grinding lysis procedure. Alternatively, a downstream component of the mitotic cascade may be irreversibly activated in those cells about to enter mitosis in the interphase populations. Nevertheless, the consistently higher level of condensing activity in mitotic extracts strongly suggests that chromatin condensation in vitro is dependent upon specific factors present at mitosis, such as MPF.

Considering the observations presented above, we suggest that our mitotic extracts faithfully reproduce several aspects of naturally occurring chromatin condensation, and provide a unique opportunity to investigate structural alterations and controls of mitotic chromosome condensation in somatic cells.

### *The Role of Topoisomerase II in Chromosome Condensation*

The classical enzymatic activity of topoisomerase II is the alteration of the topological structure of DNA brought about by transiently breaking and rejoining both strands of the DNA helix (25, 42). The need for this activity has been clearly demonstrated for the process of chromosome segregation at anaphase (4, 21, 34, 40), and it is hypothesized that the enzyme may be involved in regulating supercoiling during RNA transcription (38, 43). Genetic evidence and an in vitro inhibitor study using *Xenopus* extracts also suggest that the enzyme may be involved in chromosome condensation (30, 39). Our approach to test this hypothesis has been to in-

vestigate chromatin condensation in vitro under conditions where the level of topo II varies.

Since topo II is present in both purified nuclei and mitotic extracts, both sources of the protein must be considered. Immunodepletion of topo II from the mitotic extract does not affect chromatin condensation when nuclei purified from actively growing tissue culture cells are used as substrates. These nuclei have high levels of endogenous topo II that therefore must be capable of fulfilling the need for the enzyme, if such a need exists.

We have taken advantage of the erythroid lineage in the embryonic chick, where topo II levels decrease dramatically during development, to examine the dependence of condensation on the presence of endogenous topo II. The nuclei from primary circulating erythroblasts rapidly lose topo II as the cells cease proliferation during the developmental process (16). Using these nuclei as substrates of the in vitro chromatin condensation reaction, we have demonstrated that the ability of the interphase chromatin to condense into discrete chromosome-like structures varies with topo II levels in the purified nuclei.

Even though topo II levels correlate with the ability of the interphase chromatin to condense into discrete regions, it remains possible that the inability of late-stage erythrocyte nuclei to undergo this process is due to some as yet unidentified nuclear protein lost during erythroid development or other overall rearrangements in nuclear structure. To distinguish between these possibilities, we have attempted to restore the formation of discrete condensed regions by adding purified topo II back to the late stage chick erythrocyte nuclei. Although we have used several different sources of topo II including enzyme purified from calf thymus, HeLa cells, T2 phage, and chicken we have been unable to consistently restore the formation of discrete chromosome-like structures.

We have considered two possible explanations for these results: other proteins lost during erythropoiesis may be exclusively required, or required in combination with topo II to restore chromosome formation. Alternatively, topo II may be all that is required for chromosome formation, but the enzyme may not function properly, unless it is incorporated into an appropriate nuclear structure (possibly at the base of chromatin loop domains). Such a structure may not be easily formed simply by adding the enzyme back to the purified nuclei in these somatic cell extracts. For example, formation of this hypothetical structure might require the presence of topo II during a specific stage of the cell cycle such as DNA replication.

### *There Appear to Be Two Aspects of Chromatin Condensation In Vitro*

Interestingly, the loss of topo II does not prevent condensation of the interphase chromatin into a more compact volume. Thus it appears that overall chromosome condensation may involve two different processes, compaction of the chromatin and resolution of the individual chromosomes. The chromatin compaction process may be mediated by histone phosphorylation, which has long been known to correlate with the timing of mitosis.

Our results with 7- and 9-d erythrocyte nuclei suggest that topo II is not required for the compaction process but may be required for the resolution process. A role for topo II in the resolution process makes functional sense considering the basic strand breakage–rejoining activity of the enzyme. However, the 9-d erythrocyte nuclei used in this assay are arrested in $G_0$. Therefore, the resolution process required for this condensation is not the same as the post-replicative resolution of sister chromatids that occurs at anaphase (4, 21, 34, 40). The mechanism of this chromatin resolution potentially mediated by topo II is unclear. But given that the addition of soluble active enzyme does not restore this process, it is interesting to speculate that topo II must be incorporated into an appropriate macromolecular structure to function in this capacity.

In the future we hope to use our in vitro chromatin condensation system to help clarify the chromatin resolution process, by searching for other factors that might be needed to restore the formation of discrete condensed regions in late stage chick erythrocytes. Furthermore, the identification of two aspects of chromatin condensation (compaction and resolution) should allow us to more clearly define roles for events, such as histone phosphorylation, in chromosome condensation.

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*Note Added in Proof*: The failure of 9-d erythrocyte nuclei to condense is not due to inability of the extracts to solubilize the lamina. When these nuclei are incubated in the extract, 74% of lamin B2 is solubilized (as judged by quantitation of immunofluorescence).

We have recently developed a reproducible protocol by which highly purified topo II is able to restore the condensation phenotype to 9-d (and later) erythrocyte nuclei (Wood, E., M. Muller, and W. Earnshaw, unpublished results). This offers strong support for the conclusion that topo II is required for the in vitro condensation process.

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