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Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle

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ABSTRACT **We have utilized antibody probes to examine the expression of DNA topoisomerases I and II and chromosome scaffold protein Sc-2 in normal and transformed cells. Neither topoisomerase I nor Sc-2 shows significant fluctuations in content or stability across the cell cycle. In contrast, topoisomerase II undergoes significant cell cycle-dependent alterations in both amount and stability. As cells progress from mitosis into G_1, much of the topoisomerase II is degraded. During the first 2 hr of G_1, the half life of topoisomerase II is decreased from that measured in asynchronous cell populations by a factor of 7. This suggests that the chromosome condensation/decondensation cycle is coupled to a parallel cycle of synthesis and degradation of topoisomerase II. In control experiments, we also found that the half-life of topoisomerase II is shorter in normal cells than in transformed cells by a factor of 4. Since the number of copies of topoisomerase II per cell is also lower in normal cells, this suggests that control of topoisomerase II stability is altered upon transformation. The stability of topoisomerase I and Sc-2 does not differ significantly between normal and transformed cells.**

Topoisomerases are enzymes that regulate the superhelical density of DNA by transiently nicking either one (type I) or both (type II) strands of the DNA helix (reviewed in ref. 1). Much interest has focused on determining the functional role(s) of the two topoisomerases within eukaryotic cells, where it has been suggested that they may be involved in many aspects of DNA metabolism, including transcription, replication, recombination, and chromosome segregation at mitosis (reviewed in ref. 1). Recent experiments suggest that topoisomerase II may also be a structural component of the nucleus and mitotic chromosomes. Use of specific antibody probes reveals that topoisomerase II is a prominent component of both the mitotic chromosome-scaffold and the interphase nuclear-matrix fractions (2–4). Topoisomerase II is localized to a restricted axial domain at the base of chromatin loops within metaphase chromatids (5). The importance of topoisomerase II for mitotic chromosome function is indicated by analysis of temperature-sensitive *top2* mutations in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. At the nonpermissive temperature, these mutant yeast cells die in anaphase of mitosis as they attempt (unsuccessfully) to disjoin sister chromatids (6–8).

Topoisomerase I is apparently not a structural protein, as it is extractable from chromatin at relatively low ionic strength (9) and is distributed throughout the large ≈100-kilobase (kb) chromatin loops of the mitotic chromosome (5). In addition, topoisomerase I is not essential for growth in yeast (6, 10, 11).

Assays of topoisomerase II activity both in cell-free extracts and *in vivo* suggested that enzyme levels are greater in proliferating cells than in their quiescent counterparts (12–17). Quantitative immunological methods demonstrated that topoisomerase II is rapidly lost upon cessation of mitotic activity in either immature erythroblasts or myoblasts (18), thus confirming that the enzyme is a specific and sensitive marker for cell proliferation. Topoisomerase I, on the other hand, is present in many different quiescent cell types (12, 18).

The disappearance of topoisomerase II upon the cessation of mitotic activity suggested to us that the transition from mitosis to interphase might be accompanied by facilitated degradation of this protein. Therefore, we have used antibody probes to examine the expression and stability of the enzyme across the cell cycle, with particular emphasis on the period surrounding mitosis. As controls, we performed parallel studies on topoisomerase I and on Sc-2, a 135-kDa polypeptide that is the second most abundant component of the mitotic chromosome-scaffold fraction (19). The results indicate that the transition from mitosis into the subsequent G_1 phase is accompanied by a dramatic decrease in the stability of topoisomerase II (but not topoisomerase I or Sc-2). These observations suggest that degradation of topoisomerase II may be intimately associated with the process of chromosome decondensation.

MATERIALS AND METHODS

Cell Cultures. MSB-1 cells (chicken lymphoblastoid cells; doubling time = 14.5 hr) were grown in suspension culture in RPMI 1640 medium (GIBCO) containing 5% (vol/vol) calf serum (HyClone, Logan, UT). Chicken hepatoma (line 249) cells (doubling time = 19 hr) were grown attached to plastic tissue culture dishes in D/FBS medium [Dulbecco's modified Eagle's medium (DME medium; GIBCO) containing 10% fetal bovine serum (HyClone)]. Chicken embryo fibroblasts [doubling time = 28 hr in D/FBS medium containing 5% embryo extract (20) and 1% chicken serum (GIBCO)] were prepared from 9-day-old chicken embryos and used within 1 week.

Determination of Protein Levels. Cells were solubilized by sonication in $NaDodSO_4$/PAGE sample buffer (50 mM Tris·HCl, pH 6.8/15% sucrose/2 mM EDTA/3% $NaDodSO_4$/ 20 mM dithiothreitol). An equal number of cells from each fraction or time point was loaded per lane of replicate 10% polyacrylamide gels (21). One gel was stained with Coomassie blue to measure relative protein content. The other gels were transferred electrophoretically to nitrocellulose for immunoblotting analysis (22, 23) by utilizing two polyclonal guinea pig sera recognizing topoisomerase II (2) and Sc-2 (M.M.S.H. and W.C.E., unpublished data) and a human Scl-70 autoimmune serum cross-reactive with chicken topoisomerase I (9). Relative protein content was determined either by pyridine elution of the protein-bound Coomassie blue (24) or by densitometric scanning of the lanes (Zeineh Soft Laser Scanning Densitometer, Biomed Instruments, Fullerton, CA). Antigen levels were determined as described (18).

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Centrifugal Elutriation. MSB-1 cells in logarithmic phase were fractionated by centrifugal elutriation by using a Beckman JE-6B elutriator rotor mounted in a J2-21 centrifuge (25) as described (26). Fractions were analyzed for size and number of cells with a Coulter Counter. Cell-cycle position was determined by flow cytometry with an EPICS V electronically programmable individual cell sorter (Coulter) in association with MDADS Graphics display (Coulter). We utilized the ratio of the penultimate fraction to the second fraction from the elutriator to determine the increase in protein content and antigen levels.

Mitotic Release. Mitotic cells were obtained by selective mechanical detachment from exponentially growing subconfluent cultures of 249 cells in T-150 flasks (27). The first shake-off was discarded; the next six shake-offs (performed at 15-min intervals) were pooled for the mitotic release. Detached cells were pelleted at 1000 × *g* and resuspended in cold D/FBS medium containing 20 mM Hepes (pH 7.4). The final mitotic cell suspension was pelleted, resuspended in warm D/FBS medium containing 20 mM Hepes (pH 7.4) and plated on 60-mm tissue culture dishes. The mitotic index was determined by phase microscopy. An aliquot of this mitotic sample was immediately lysed for gel analysis. At 45 min after mitotic release, unattached cells (presumed dead or recovering more slowly) were aspirated from the culture dishes, and fresh medium was returned to the dishes. At each time point, the cells were dissociated from the culture dish with 0.1% trypsin in phosphate-buffered saline and diluted in cold D/FBS medium. The cells were counted (the mitotic index was also determined), pelleted, and lysed in $NaDodSO_4$/PAGE sample buffer.

Determination of Half-Life. Topoisomerases I and II and Sc-2 were immunoprecipitated (18, 24) from radioactively labeled cells (below). After electrophoresis, gels were stained with Coomassie blue and subsequently fluorographed in dimethyl sulfoxide containing 2,5-diphenyloxazole (28). Radioactivity was detected by exposure of preflashed films at −70°C and quantitated by densitometry of the autoradiograms.

Chicken hepatoma (249) cells. Cells were plated on 60-mm dishes in D/FBS medium, rinsed twice with warm RPMI 1640 medium lacking methionine ($RPMI^{Met^-}$; GIBCO), and labeled with 50 μCi (1 Ci = 37 GBq) of L-[^{35}S]methionine (>800 Ci/mmol; aqueous solution, Amersham) per ml in 10% D/FBS/90% $RPMI^{Met^-}$ for 3 hr. Cells were rinsed twice with warm DME medium and incubated in D/FBS medium for the chase period (30 hr). At each of 10 time points, cells were solubilized and processed for immunoprecipitation.

Mitotic 249 cells. Cells were labeled for 3 hr with 40 μCi of [^{35}S]methionine per ml in 10% D/FBS/90% $RPMI^{Met^-}$. An equal volume of D/FBS medium was then added for 1 hr. Mitotic cells were obtained as above with two differences. (*i*) The radioactive medium was returned to the cells after each shake-off. (*ii*) Rather than plating the cell suspension, we placed aliquots into 1.5-ml tubes and kept them in a 37°C water bath for the chase period. At the desired chase times (five time points in a 2- to 3-hr chase), cells were pelleted and lysed for immunoprecipitation.

Primary chicken embryo fibroblasts. Fibroblasts were plated, pulse-labeled, chased, and processed for immunoprecipitation as above for the chicken hepatoma 249 cells.

Mitotic fibroblasts. Primary fibroblasts were labeled for 5 hr with 25 μCi of [^{35}S]methionine per ml in 25% D/FBS/75% $RPMI^{Met^-}$. A mitotic fibroblast suspension was obtained by first washing cell monolayers with phosphate-buffered saline and then incubating with phosphate-buffered saline containing 2 mM EDTA for 1 min. Mitotic cells (>70% mitotic index) were then obtained by selective detachment. The radioactivity in the mitotic cells was chased as for the transformed mitotic cells.

RESULTS

Dependence of Topoisomerase II Levels on Cell-Cycle Position. We used centrifugal elutriation to fractionate an asynchronous cell population according to cell size (i.e., cell-cycle position). Data from a typical experiment are shown in Fig. 1. Changes in topoisomerase II (Fig. 1*B*) topoisomerase I (Fig. 1*C*), and Sc-2 (Fig. 1*D*) levels were compared by quantitative immunoblotting to the increase in total cellular protein (determined by quantitation of Coomassie blue staining; see Fig. 1*A* and *Materials and Methods*). The final level of topoisomerase II relative to protein content increased 1.67-fold (±0.45; $n = 6$).

In contrast, the ratio of topoisomerase I to total protein remained essentially constant (1.01 ± 0.11; $n = 3$) as cells progressed through the cell cycle, as did the level of Sc-2 (1.08 ± 0.11; $n = 4$). Thus, the regulation of topoisomerase II expression differs from that of the two other nuclear proteins and from that of the general complement of cellular proteins.

Correlation of these data with cell-cycle position is presented in Fig. 2. When fractions with similar flow-cytometry profiles were analyzed (Fig. 2 *Lower*), it emerged that the level of topoisomerase II starts to increase just prior to or at

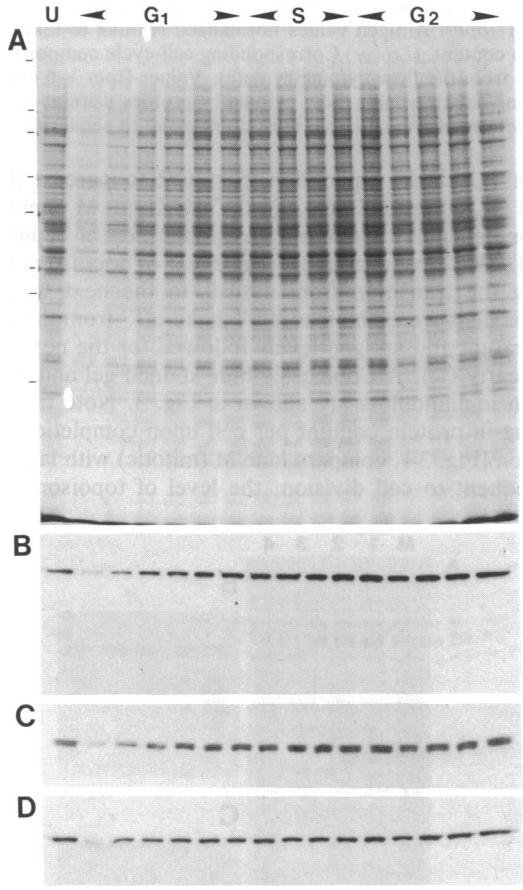

FIG. 1. Variation of chromosomal antigens across the cell cycle. An asynchronous culture of MSB-1 cells was fractionated by centrifugal elutriation. The predominant cell-cycle phase in each fraction is indicated at the top ("U" is a loading from the initial unsorted culture). Equal numbers of cells from each fraction were separated in replicate gels by $NaDodSO_4$/PAGE. (*A*) Coomassie blue-stained polyacrylamide gel. (*B–D*) Nitrocellulose transfers from gels run in parallel probed with guinea pig anti-topoisomerase II (*B*), with human autoimmune anti-topoisomerase I (*C*), and with guinea pig anti-Sc-2 (*D*). Molecular mass standards are indicated from top to bottom on the left of *A*: 200, 116, 95, 68, 60, 43, 40, and 29 kDa.

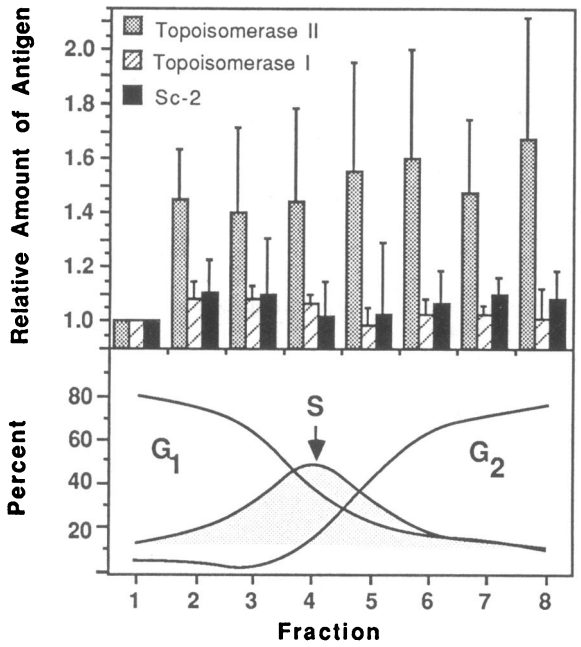

FIG. 2. Accumulation of chromosomal antigens across the cell cycle. (*Upper*) Antigen values normalized relative to total cellular protein content. (*Lower*) Corresponding cell-cycle composition (averaged from the flow-cytometry data). Values from 3–6 elutriation fractions having similar flow-cytometry profiles were averaged for each determination (the thin lines correspond to semierror bars).

the onset of DNA replication, continues to increase through S and G_2 phase, and peaks in the late G_2 + M population.

Completion of Mitosis and the Specific Loss of Topoisomerase II. We next examined the levels of topoisomerase II during the transition from mitosis to the next G_1 phase. Mitotic cells were obtained by "shake-off" from the culture substratum and antigen levels followed for the next 3–4 hr (i.e., early G_1). A Coomassie blue-stained gel and its companion immunoblots are shown in Fig. 3. Note the abrupt halving in protein content per cell upon completion of cell division [Fig. 3*A*, compare lane M (mitotic) with lanes 1–4]. Subsequent to cell division, the level of topoisomerase II continued to decline (Fig. 3*B*), while that of topoisomerase I remained relatively constant (Fig. 3*C*).

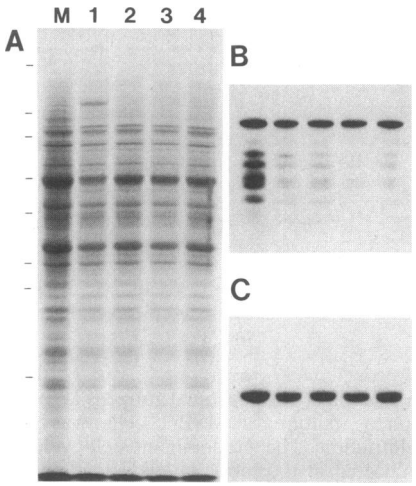

FIG. 3. Variation in topoisomerase I and II levels after mitosis. Equal numbers of cells were loaded per lane of replicate polyacrylamide gels. (*A*) Coomassie blue-stained gel. Lanes: M, mitotic cells; 1–4, time points after mitosis (45, 90, 135, and 180 min). (*B* and *C*) Nitrocellulose transfers probed with guinea pig anti-topoisomerase II (*B*) and human anti-topoisomerase I (*C*). Molecular mass standards indicated on the left of *A* are the same as those used in Fig. 1.

The compiled results of the mitotic release experiments are presented in Fig. 4. The starting mitotic index was 89.4 ± 6.9% (n = 4). By 90 min after mitotic release, nearly all cells had completed cell division [only 5.4 ± 1.1% (n = 3) were in telophase]. Subsequent time points contained even fewer (1–2%) telophases. In the figure, the dotted line indicates the anticipated antigen level, given equivalent partitioning to the two daughter cells after mitosis (based on the observed mitotic index). Experimental time points are expressed as a percentage of the mitotic value. Topoisomerase II rapidly deviated from the expected level; by 90 min after mitotic release, the level per cell fell to 36.4 ± 7.9% (n = 5) of that present in the mitotic cell. (Based on the mitotic index of the population, one would expect 52.5% of the mitotic antigen value at that time.) The amount of topoisomerase II per cell continued to decrease (gradually), reaching 30.6 ± 2.9% (n = 3) per cell at 3 hr after mitotic release.

In contrast, the processing of topoisomerase I after mitosis closely resembled the partitioning of total protein to the two daughter cells. At 90 min the amount of topoisomerase I per cell was 57.8 ± 2.5% (n = 3) of that in the starting mitotic population. This increased slightly [to 61.3 ± 1.6% (n = 2) at 3 hr] during these experiments.

Alteration in the Amount of Topoisomerase II Proteolysis During the Cell Cycle. The main band of topoisomerase II at 170 kDa is accompanied by a highly reproducible ladder of fragments (migrating between 80 and 150 kDa). We previously demonstrated that these are derived from the 170-kDa band by proteolysis (2). This proteolysis is likely to occur *in vivo*, as immunoblots of whole cells washed in cold phosphate-buffered saline (containing trasylol, chymostatin, leupeptin, antipain, pepstatin, and diisopropyl fluorophosphate) prior to lysis by sonication in $NaDodSO_4$/PAGE sample buffer at a variety of temperatures from 20°C to 100°C still contained these fragments (unpublished data).

Progression from G_1 to G_2 + M was accompanied by an increase in endogenous proteolysis of topoisomerase II in MSB-1 cells (Fig. 1*B*). We found 28.1 ± 2.7% (n = 4) of the total immunoreactive topoisomerase II as fragments in G_1 cells. This increased to 38.1 ± 3.6% (n = 4) in the G_2 + M population. The mitosis to G_1 transition was also accompanied by a significant change in the degree of endogenous proteolysis of topoisomerase II. Fragments constituted 61.7

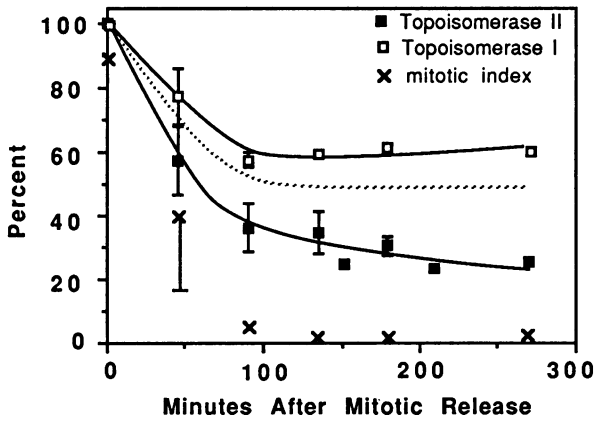

FIG. 4. Fate of topoisomerases I and II during the mitosis–G_1 transition. Values are the average of three to five independent experiments similar to that shown in Fig. 3, and the standard deviations (which in some cases are smaller than the diameter of the symbol in the graph) are shown. Antigen level is plotted on a per cell basis. The predicted amount of total protein (·····) was calculated from the average mitotic index (×) at each time point, assuming equivalent partitioning between daughter cells.

Table 1. Half-lives of nuclear proteins in hours

Nuclear proteins	Transformed cells (249)	Primary culture (CEF)
Topoisomerase II	12.0 ± 2.9 (4)	3.3 ± 0.5 (8)
Mitotic topoisomerase II	1.8 ± 0.4 (3)	1.3 ± 0.1 (2)
Topoisomerase I	15.9 ± 1.1 (2)	23.1 ± 0.8 (2)
Sc-2	16.4 ± 3.4 (4)	13.9 ± 3.8 (4)

Numbers in parentheses are the number of determinations.

± 8.7% (n = 5) of mitotic topoisomerase II in 249 cells (Fig. 3*B*). By 90 min after mitotic release, the percentage of total topoisomerase II as fragments had dropped to 48 ± 7.6% (n = 5). Although the absolute percentages differ in the two cell lines utilized, the trend of topoisomerase II proteolysis was clearly similar.

The Half-Lives of Nuclear Proteins in Transformed Cells. The results of pulse–chase experiments designed to determine the half-lives of topoisomerases II and I and Sc-2 are summarized in Table 1. An autoradiogram of [^{35}S]methionine-labeled topoisomerase II immunoprecipitated from the 249 line is presented in Fig. 5*A* (chase times in hours are given above *A*). The half-life of topoisomerase II in these cells was 12 (±2.9) hr. Topoisomerase I and Sc-2 were slightly more stable in this cell line, with half-lives of 15.9 (±1.1) hr and 16.4 (±3.4) hr, respectively. The measured half-life of topoisomerase II during the transition from mitosis to G_1 was 1.8 (±0.4) hr. Therefore, the loss of topoisomerase II associated with chromosome decondensation is apparently due to facilitated degradation during this period of the cell cycle.

Topoisomerase II in Normal Cells. Topoisomerase II was significantly less stable in primary fibroblast cultures than in the transformed cells examined. The topoisomerase II half-life in these cultures was only 3.3 (±0.5) hr (see Fig. 5*B Upper* for the autoradiogram). The rates of topoisomerase II degradation in 249 cells and embryo fibroblasts are directly compared in Fig. 5*B Lower*; note that the slope of topoisomerase II decay in normal fibroblasts is much steeper than the corresponding slope for the transformed cell line. The half-lives of fibroblast topoisomerase I and Sc-2, on the other hand, did not vary as much from those determined when using the 249 line (see Table 1).

In normal embryo fibroblasts synchronized by "shake-off," the degradation rate of topoisomerase II was greater during early G_1 [i.e., a half-life of 1.3 (±0.1) hr] than in asynchronous cultures. Therefore, chromosome decondensation in normal cells is also likely to be accompanied by a rapid turnover of topoisomerase II similar to that observed in transformed cells.

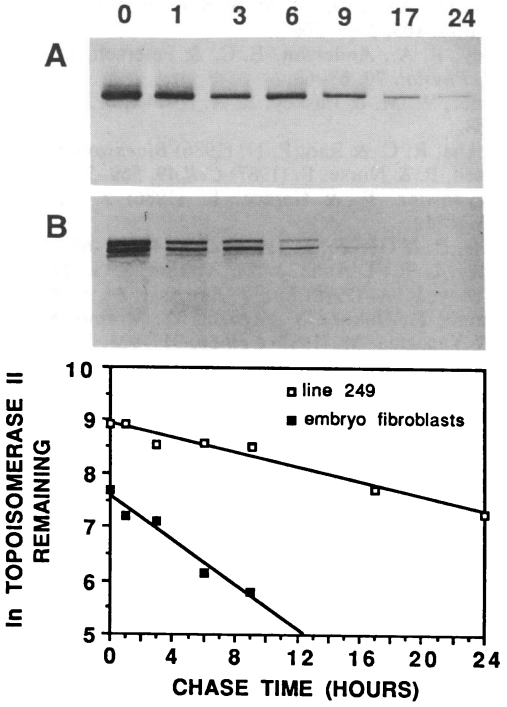

FIG. 5. Determination of the half-life of topoisomerase II in normal and transformed cells. Topoisomerase II was immunoprecipitated from radiolabeled transformed 249 cells (*A*) and normal chicken embryo fibroblasts (*B Upper*). Chase times (in hours) are given at the top of *A*; radioactive topoisomerase II remaining at each time point is plotted in *B Lower*. The top band in *B Upper* is also precipitated by preimmune serum and was excluded from the measurements.

DISCUSSION

Topoisomerase II accumulates throughout the cell cycle in transformed cells with the level of enzyme increasing >3-fold per cell, peaking prior to mitosis. Topoisomerase I and Sc-2, on the other hand, double in amount, thus behaving like the bulk of cellular proteins. The topoisomerase II accumulation prior to mitosis is balanced by a sharp decrease in the level of the antigen during early G_1 phase. This rapid decline in the amount of topoisomerase II apparently is accomplished by decreasing the stability of the enzyme. In contrast, the stability of topoisomerase I appears not to vary during mitosis.

Control of Chromosome Condensation. Current models suggest that the structural reorganization of cells during mitosis is brought about primarily by protein phosphorylation events that may result from a "cascade" of kinase activities (29, 30). The nuclear lamins become reversibly phosphorylated during mitosis, and lamina disassembly results (24, 31); lamina reassembly is probably regulated by dephosphorylation (32). Thus, the lamins may be used in several rounds of nuclear disassembly/assembly. In addition, phosphorylation of histones H1 and H3 correlates with chromosome condensation during prophase (33).

Our results suggest that the dramatic structural changes that accompany mitosis are dictated not only by reversible posttranslational modifications but also by changes in the relative levels of constituent proteins. Thus, the majority of topoisomerase II molecules are not reused for multiple cell cycles. In fact, mitotic chromosome assembly and topoisomerase stability may be intimately related. The cycle of synthesis and degradation of topoisomerase II correlates well with the chromosome condensation/decondensation cycle. This is consistent with previous results suggesting a role for topoisomerase II in establishing and/or maintaining the condensed mitotic chromosome architecture (2, 5). Recent analysis of a cold-sensitive *top2* mutation in *S. pombe* confirms that topoisomerase II activity is required for the final stages of chromosome condensation (34).

The observed sharp decrease in the half-life of topoisomerase II in postmitotic cells may be a consequence of disassembly of the mitotic chromosome scaffold during chromosome decondensation. A correlation between assembly state and stability has been shown (35) for α- and β-spectrin in the erythrocyte cytoskeleton. Alternatively, decreased stability of topoisomerase II may contribute to the process of chromosome decondensation.

Even though the level of topoisomerase II protein is regulated in a cell-cycle-specific manner, posttranslational modifications of the enzyme may also contribute to the regulation of its activity and/or stability. Topoisomerase II is phosphorylated *in vitro* by casein kinase II (36) and protein kinase C (37), leading to a 3-fold increase in enzymatic

activity. *In vivo* phosphorylation has yet to be demonstrated. If topoisomerase II were phosphorylated during mitosis, one would expect an ≈10-fold increase in activity (taking into account the increase in enzyme content prior to mitosis). In fact, 4- to 15-fold more topoisomerase II activity was detected in mitotic HeLa cells than in an S-phase population (38). Phosphorylation may be responsible for rapid changes in activity in response to intracellular signals, whereas alterations in the amount of protein may be required for the gross structural reorganization of the genome during mitosis.

Examination of the data of ref. 36 suggests that phosphorylation of topoisomerase II occurs mainly on the 170-kDa form and much less on the proteolytic fragments. Since several of these fragments are larger than a catalytically active fragment previously isolated from bovine thymus (39), this raises the possibility that a combination of proteolysis and phosphorylation might generate a repertoire of "isoforms" that may have different specific activities, and possibly different biological roles (see also ref. 40). The observation that the pool of fragments is maximal at mitosis suggests, for example, that the 170-kDa form might be responsible for sister chromatid disjunction (6–8) and that the fragments might be involved in structural interactions. Posttranslational modifications may also modulate the structural interactions of topoisomerase II with other chromosomal components such as Sc-2.

Topoisomerase II in Normal and Transformed Cells. Transformed cells have a pool of topoisomerase II molecules roughly 5-fold larger than that of their normal counterparts. Increased topoisomerase II activity, as assayed by drug-induced DNA cleavage or DNA decatenation, has also been associated with the transformed phenotype (16, 17).

We now have found that the half-life of topoisomerase II is shorter in normal primary chicken embryo fibroblasts by almost a factor of 4 than in a transformed chicken hepatoma cell line. This half-life varies as a function of the cell cycle in normal as well as transformed cells, being dramatically decreased during the mitosis–G_1 transition. It is possible that topoisomerase II is entirely degraded during G_1 phase in normal cells and only resynthesized at (or just prior to) the onset of DNA replication, as was seen in the entry of G_0 lymphocytes into the cell cycle (18). Failure to resynthesize topoisomerase II could be associated with exit from the cell cycle. Therefore, it is tempting to speculate that the expression of topoisomerase II could be linked to the expression of early G_1 genes in association with commitment to enter the cell cycle.

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