

Cell Cycle-coupled Relocation of Types I and II Topoisomerases and Modulation of Catalytic Enzyme Activities

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Abstract. We visualized DNA topoisomerases in A431 cells and isolated chromosomes by isoenzyme-selective immunofluorescence microscopy. In interphase, topoisomerase I mainly had a homogeneous nuclear distribution. 10–15% of the cells exhibited granular patterns, 30% showed bright intranucleolar patches. Topoisomerase II isoenzymes showed spotted (α) or reticular (β) nuclear patterns throughout interphase. In contrast to topoisomerase II α , topoisomerase II β was completely excluded from nucleoli. In mitosis, topoisomerase II β diffused completely into the cytosol, whereas topoisomerases I and II α remained chromosome bound. Chromosomal staining of topoisomerase I was homogeneous, whereas topoisomerase II α accumulated in the long axes of the chromosome arms and in the centriols. Topoisomerase antigens were 2–3-fold higher in mitosis than in interphase, but specific activities of topoisomerase I and II were reduced 5- and 2.4-fold, respec-

tively. These changes were associated with mitotic enzyme hyperphosphorylation. In interphase, topoisomerases could be completely linked to DNA by etoposide or camptothecin, whereas in mitosis, 50% of topoisomerase II α escaped poisoning. Refractoriness to etoposide could be assigned to the salt-stable scaffold fraction of topoisomerase II α , which increased from <2% in G₁ phase to 48% in mitosis. Topoisomerases I and II β remained completely extractable throughout the cell cycle. In summary, expression of topoisomerases increases towards mitosis, but specific activities decrease. Topoisomerase II β is released from the heterochromatin, whereas topoisomerase I and II α remain chromosome bound. Scaffold-associated topoisomerase II α appears not to be involved in catalytic DNA turnover, though it may play a role in the replicational cycle of centriols, where it accumulates during M phase.

IN the cell, DNA topology is regulated and controlled by ubiquitous enzymes known as topoisomerases, which break and reseal the polyphosphate backbone of the DNA and pass other strands of DNA through the transient gaps (16, 19, 39, 40, 59–61). There are two types of DNA topoisomerases with differing properties. Type I topoisomerases can alter the pitch of DNA double helices by cutting one DNA strand and allowing passage of the complementary strand through the transient nick (19). Type II topoisomerases require ATP hydrolysis for catalytic activity and alter DNA topology by creating transient double strand breaks through which a second intact double helix is passed (40). Topoisomerase function is re-

quired for replication, transcription (29, 67), recombination (32, 50), and repair (27, 31, 51) of DNA but also for chromosome (de)condensation (1, 20, 66) and sister chromatid segregation (13, 37). To date it is not completely clear how these multiple functions are assigned to the various types and isoforms of topoisomerases. In particular, it is unknown why mammals possess two isoforms of type II topoisomerases, α and β , which are encoded by separate genes (24, 56), whereas insects and fungi have only one isoform (62). Topoisomerase II has been identified as the major nonhistone protein which links the basis of the radial chromatin loops to the central axis of the mitotic chromosome fiber (44). In mammals, the enzyme is concentrated in the long axes of the chromosomes (48, 53), whereas in insects it is distributed uniformly in the whole chromosomal fiber. Moreover, in insects, 70% of the enzyme dissociates from the chromosomes during the mitotic cycle and diffuses into the cytosol (52). It has also been shown that the bulk of topoisomerase II can be extracted

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from reconstituted chromosomes of *Xenopus laevis* oocytes without disrupting their morphology (20).

To reconcile these controversial findings, it has been hypothesized that topoisomerase II might have a structural as well as a diffusible enzymatic role in the formation of condensed chromosomes (1), and that in mammals, these two functions could correspond to the two isoforms of topoisomerase II (44). However, the data available on the nuclear localization of topoisomerase II α and β in mammalian cells equivocally support this concept. One set of studies carried out with polyclonal peptide antibodies directed against unique epitopes of the two isoenzymes showed that both type II topoisomerases diffused into the cytosol during mitosis, whereas mitotic chromosomes were not stained (42). Other studies carried out with monoclonal antibodies claimed that topoisomerase II β is exclusively located in the nucleoli and belongs to the structural elements of the insoluble nucleolar remnant, thus excluding its role in diffusible topoisomerase II activity during mitotic chromosome condensation (68, 69). However, the monoclonal antibodies used in the latter studies for detecting topoisomerase II β , as we observed, did not actually bind to recombinant purified human topoisomerase II β , as will be discussed later. This observation led us to reinvestigate the subcellular localization of human topoisomerase isoenzymes using a new set of monoclonal and polyclonal antibodies directed against human DNA-topoisomerases I, II α , and II β , different from those used in previous studies. Making use of purified recombinant human topoisomerases produced in *Saccharomyces cerevisiae*, we could clearly establish the specificity of these antibodies. Moreover, similar results were obtained with monoclonal antibodies and peptide antibodies targeting the same topoisomerase by different epitopes. Our results confirm and complete previous reports regarding the localization of topoisomerase I and II α (12, 14). However, the nuclear localization of topoisomerase II β observed here is different from that previously reported (42, 68, 69). We find that topoisomerase II β is not located inside the nucleoli but is exclusively present in the extranucleolar nucleoplasm throughout interphase. In mitosis it is not part of the chromosomal scaffold but becomes released from the chromatin, whereas the whole cellular complement of topoisomerase II α localizes to the central axis of the chromosomes. In contrast to previous reports on the cellular localization of topoisomerase II isoenzymes, our findings are in good agreement with concepts currently held on the differential role of topoisomerase II α and β in mitotic cells (44).

Materials and Methods

Cells

Human A431 epidermoid cells (ATTC No. 1555, American Type Culture Collection, Rockville, MD) were grown in liquid culture (DME with FBS 10% (vol/vol), 10 g/liter penicillin/streptomycin, 1% l-glutamine) in a humidified atmosphere containing 5% (vol/vol) CO₂. Cells were routinely checked to be free of mycoplasmas by immunoassays and cultural analysis. For indirect immunofluorescence, cells were grown on microscope slides. Cells were trapped in mitosis by treatment with 0.26 μ M demecolcine (Sigma Chemical Co., Deisenhofen, Germany) for 16 h and continued to grow in a synchronized manner for one cell cycle after washing out demecolcine. Analysis of cell cycle phases was performed on cells detached from

the substratum by trypsinization, fixed with 70% ethanol, and permeabilized with 0.1% Nonidet P-40. DNA was stained with bisbenzimidazole (0.1 μ g/ml), and cellular DNA content was analyzed using a Partec flow cytometer equipped with a xenon lamp and trout erythrocytes as a DNA standard. Mitotic indices were scored by counting 200 cells stained with bisbenzimidazole under the fluorescence microscope.

Antibodies and Recombinant Human Topoisomerases

Topoisomerase I. We used γ -globuline fractions of human Scl-70 auto-antibodies (Dunn Laboratories, Asbach, Germany) and rabbit antibodies generated against peptides homologous to the NH₂ (residues 1–17) or COOH terminus (residues 745–765) of human topoisomerase I (Genosys Biotechnologies, Cambridge, UK). Human topoisomerase I was heterologously expressed in *S. cerevisiae* and purified, as described previously (7).

Topoisomerase II α . We have previously established that the mouse monoclonal antibody directed against the proliferation-associated nuclear antigen Ki-S1 is specific for a COOH-terminal epitope of human topoisomerase II α and does not cross-react with topoisomerase II β (6). In the same study we have demonstrated that peptide antibodies generated against unique NH₂- (residues 1–15) or COOH-terminal peptides (residues 1512–1530) of human topoisomerase II α are specific for human topoisomerase II α and do not cross-react with human topoisomerase II β . Heterologous production of human topoisomerase II α was also described in 6.

Topoisomerase II β . Production and characterization of the mouse monoclonal antibody 3H10 has recently been described in (26). Rabbit antibodies raised against peptides homologous to a unique COOH-terminal sequence (residues 1611–1621) of human topoisomerase II β have previously been characterized (6). Recombinant human topoisomerase II β purified from *S. cerevisiae* was a kind gift (Dr. Ole Westergaard, Department of Molecular and Structural Biology, University of Aarhus, DK).

Indirect Immunofluorescence Microscopy

Cells grown on microscope slides were fixed with formaldehyde (3.7% in PBS) for 10 min at 5°C and subsequently permeabilized with Nonidet P-40 (0.01% in PBS) for 5 min at 4°C. After washing with PBS, cells were blocked for 1 h at 20°C with PBS containing 5% standard goat serum and 1% BSA. Subsequently, cells were incubated for 1 h at 20°C with primary antibodies diluted 1:400 (Ki-S1), 1:2,000 (Scl-70), or 1:250 (3H10) in PBS containing 1% BSA. After washing, bound antibodies were visualized by incubation for 1 h at 20°C with goat anti-human, -mouse, or -rabbit Fab₂-fragments, which were labeled with CY3 (Dianova GmbH, Hamburg, Germany) and diluted 1:1,000 in PBS containing 1% BSA and 1% standard goat serum. After washing with PBS, DNA was counterstained with 1 μ g/ml of bisbenzimidazole (Hoechst 33258) in PBS for 5 min at 20°C. Stained cells were mounted in antifade solution (PBS containing 1.5% N-propyl-galate and 60% glycerol) and examined at 480 or 1200 magnification using a Leitz DM epifluorescence microscope coupled to a cooled CCD camera (PM512; Photometrics Ltd., Tuscon, AZ). Camera control and image acquisition were done with an Apple Quadra 800 computer equipped with imaging software from IPLabSpectrum. Fluorophores were selectively imaged with filters specially prepared as described by Pinkel et al. (43). Signals from bisbenzimidazole and CY3 were visually distinct and readily identifiable by inspection using appropriate filters.

Western Blot and Immuno-band Depletion Assay

Cells were cultured with or without inhibitors, followed by trypsinization (also in the presence or absence of inhibitors), sedimentation of detached cells (1,000 g, 5 min, 4°C), subsequent lysis in 1% SDS for 5 min at 90°C, and mechanical DNA shearing with a syringe. Samples equivalent to 5 \times 10⁵ cells were subjected to SDS-polyacrylamide (8%) gel electrophoresis. Proteins in the gel were electrophoretically transferred to PVDF membranes (Immobilon P; Millipore Corp., Bedford, MA) by the semi-dry method using 70 mM CAPS buffer, pH 11. Immunostaining of immobilized proteins with various topoisomerase antibodies was carried out at room temperature for 1 h using peroxidase-labeled goat secondary antibodies (Dianova GmbH), and the ECL system. Migration distances of immunostained protein bands were compared to those of rabbit muscle myosin (212 kD), α_2 -macroglobulin from bovine plasma (170 kD), β -galactosidase from *Escherichia coli* (116 kD), human transferrin (76 kD), and bovine liver glutamic dehydrogenase (53 kD). Band-depletion phenomena were quantified by comparing the optical densities (determined with a video

densitometer; Froebel, Wasserburg, Germany) of immunobands located on the same gel and film after subtracting the background value of the x-ray film.

Determination of Extractable Nuclear Topoisomerase Activities

Cells were detached by trypsinization, and cell nuclei were isolated and extracted with 350 mM NaCl, as described in (5). For activity assays, nuclear extracts were serially diluted into a final volume of 40 μ l of 10 mM BisTrisPropane, pH 7.9, containing 10 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM EDTA, and 0.03 mg/ml BSA. Topoisomerase I activity was assessed by relaxation of 250 ng pBR 322 plasmid DNA in the presence of 1 mM Na₃VO₄. Under these conditions, topoisomerase II activity was completely inhibited, as demonstrated by the absence of DNA-unknotted activity. Topoisomerase II activity was measured by unknottedting of 250 ng of bacteriophage P4 knotted plasmid DNA in the presence of 1 mM ATP (23). Incubation at 37°C for 30 min was terminated by addition of 1% SDS. Samples were then digested with 1 mg/ml proteinase K at 37°C for 30 min. Gel electrophoresis was performed at 0.4 V/cm for 12 h in 1% agarose gels with TAE buffer. Gels were stained with 0.5 μ g/ml ethidium bromide after electrophoresis. Fluorescence of ethidium bromide in the gels (excitation 302 nm, emission >600 nm) was documented by Polaroid photography. The relative amounts of relaxed and supercoiled pBR322 DNA or unknotted P4-DNA were determined by video-amplified fluorescence intensity measurements of the respective DNA bands in each lane of the gel using a video-densitometer. One unit of topoisomerase I was defined as the amount of enzyme that relaxes 250 ng of pBR 322 DNA by 50% under the given conditions. One unit of topoisomerase II was defined as the amount of enzyme catalyzing a halfmaximal increase in the band of unknotted P4 DNA.

Determination of Nuclear Topoisomerase Antigen Levels

5×10^5 nuclei purified from synchronized cells in G₁, S, or G₂ phase, cells trapped in mitosis by demecolcine, or a normal logarithmic cell culture were lysed with hot SDS, sheared with a syringe, and subjected to Western blotting. Blots were probed with antibodies specific for human topoisomerase I, II α , or II β , as described under Western blot and Immuno-band Depletion Assay. For quantitative determination of protein expression, the intensities of the immunoreactive bands were measured by video-densitometry of the x-ray films using a video densitometer (Froebel) and compared to those obtained with defined amounts of purified recombinant topoisomerases analyzed in the same gel and on the same x-ray film.

Preparation of Metaphase Chromosomes

Cells were treated with colcemid (0.1 μ g/ml) for 1 h, harvested, sedimented for 5 min at 500 g, swelled with 0.075 M KCl for 10 min at 7°C, and sedimented again for 5 min at 500 g. Cells were fixed 3 times in freshly prepared methanol/acetic acid (3:1, vol/vol) for 3 min, spotted onto microscope slides, and air dried. Immunostaining was done as described for whole cells.

Phosphatase Treatment and Analysis of Molecular Weight Shifts

Nuclei from 5×10^7 cells trapped in mitosis by demecolcine, or from a normal logarithmic cell culture were extracted with 800 mM NaCl. Extracts were precipitated with 3 M ammonium sulfate. Precipitates were renatured with 200 mM diethanolamine, pH 9.9, containing 2 mM MgCl₂ and 1 mM PMSF, and incubated with 30 U of alkaline phosphatase from calf intestine (Boehringer Mannheim GmbH, Mannheim, Germany) for 90 min at 37°C. Controls were incubated with an equivalent amount of phosphatase storage buffer without phosphatase. Subsequently, proteins were precipitated with 15% (wt/vol) of trichloroacetic acid for 10 min at 37°C. Precipitates were washed with an equal volume of acetone cooled to -20°C, dissolved in SDS loading buffer, and finally subjected to SDS-gel electrophoresis in 5.5% polyacrylamide gels and Western blotting.

Results

Specificity Controls of Topoisomerase Antibodies

Isoenzyme specific detection of human topoisomerases I, II α , and II β by Western blotting and indirect immunofluorescence microscopy was established using purified recombinant human topoisomerases produced in yeast as test antigens. Fig. 1 compares Western blots of purified enzymes and of whole A431 cell lysates probed with antibodies directed against various epitopes of topoisomerases I, II α , or II β . Fig. 2 shows specificity controls of immunostaining of topoisomerases I, II α , or II β in fixed and permeabilized cells, using heat-inactivated (60°C, 5 min), purified recombinant human topoisomerases for preabsorption of the antibodies.

Topoisomerase I was detected by peptide antibodies directed against the termini of the enzyme and by human Scl-70 autoantibodies. In Western blots both peptide antibodies as well as the Scl-70 autoantibodies reacted with purified recombinant topoisomerase I (Fig. 1, lanes 2–4), and in lysates of whole A431 cells, a single band was stained (Fig. 1, lanes 5–7) which had a size identical to that of purified human topoisomerase I visualized by Coomassie blue staining (Fig. 1, lane 1). Immunofluorescent images of A431 cells stained with Scl-70 autoantibodies (Fig. 2 a) could be completely blocked by preincubating the antibodies with 1 μ g of purified and heat-inactivated human topoisomerase I (Fig. 2 b).

Topoisomerase II α was detected by peptide antibodies directed against the COOH terminus of the enzyme and by the mouse monoclonal antibody Ki-S1 also recognizing a unique COOH-terminal epitope of human topoisomerase II α (6). In Western blots, peptide antibodies and the Ki-S1 monoclonal antibody reacted with recombinant human topoisomerase II α produced in *S. cerevisiae* (Fig. 1, lanes

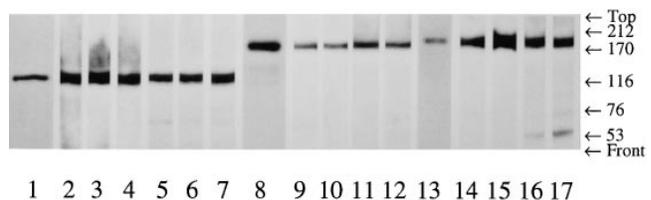


Figure 1. Reactivity of topoisomerase antibodies in Western blotting. 50 ng of purified recombinant human topoisomerase I (lanes 2–4), II α (lanes 9 and 10), or II β (lanes 14 and 15) or 5×10^5 A431 cells lysed in hot SDS (lanes 5–7, 11, 12, 16, and 17) were separated by SDS-PAGE and blotted onto Immobilon-P membranes. Lanes 1, 8, and 13 show Coomassie blue staining of 300 ng of purified topoisomerases I, II α , and II β , respectively. Blot membranes were probed with antibodies, as follows. Lanes 2 and 5: Scl-70, 1:2,000; lanes 3 and 6: rabbit anti-peptide directed against human topoisomerase I-COOH terminus, 1:5,000; lanes 4 and 7: rabbit anti-peptide directed against human topoisomerase I-NH₂ terminus, 1:1,000; lanes 9 and 11: Mouse monoclonal antibody Ki-S1, 1:500; lanes 10 and 12: rabbit anti-peptide antibody directed against human topoisomerase II α -COOH terminus, 1:5,000; lanes 14 and 16: Mouse monoclonal antibody 3H10, 1:800; lanes 15 and 17: Rabbit anti-peptide antibody directed against human topoisomerase II β -COOH terminus, 1:5,000. Strips represent the whole running distance of the gel, excluding the stacking portion.

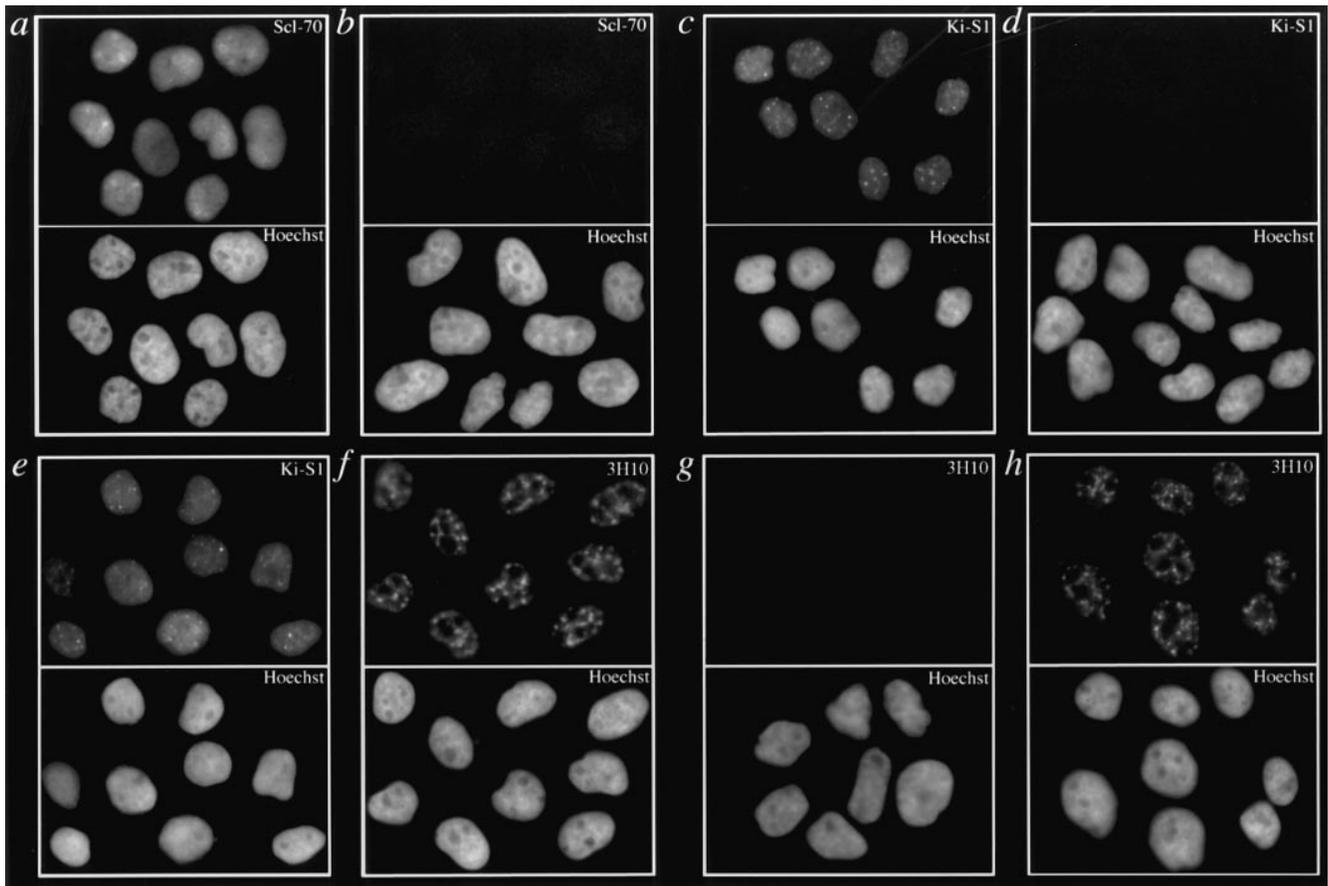


Figure 2. Specificity controls of indirect immunofluorescence microscopy of topoisomerases. A431 cells were grown on microscope slides, fixed, permeabilized, and double stained with topoisomerase antibodies and bisbenzimidazole (Hoechst Frankfurt, Germany). Images of immunofluorescence (*top*) and DNA (*bottom*) are paired. (*a*) Immunostaining of topoisomerase I with Scl-70 autoantibodies; (*b*) as *a*, but Scl-70 autoantibodies were preincubated with 1 μ g of heat-inactivated (60°C, 5 min) human topoisomerase I for 1 h at 20°C; (*c*) Immunostaining of topoisomerase II α with Ki-S1 mouse monoclonal antibody; (*d*) as *c*, but Ki-S1 antibody was preincubated with 1 mg of heat-inactivated (60°C, 5 min) human topoisomerase II α for 1 h at 20°C; (*e*) as *c*, but Ki-S1 antibody was preincubated with 1 μ g of heat-inactivated (60°C, 5 min) human topoisomerase II β for 1 h at 20°C; (*f*) immunostaining of topoisomerase II β with 3H10 mouse monoclonal antibody; (*g*) as *f*, but 3H10 antibody was preincubated with 1 μ g of heat-inactivated (60°C, 5 min) human topoisomerase II β for 1 h at 20°C; (*h*) as *f*, but 3H10 antibody was preincubated with 1 mg of heat-inactivated (60°C, 5 min) human topoisomerase II α for 1 h at 20°C.

9 and 10). In lysates of whole A431 cells, both antibodies stained a single band of 170 kD (Fig. 1, lanes 11 and 12) identical in size to purified human topoisomerase II α visualized by Coomassie blue staining (Fig. 1, lane 8). Immunofluorescent images of A431 cells stained with Ki-S1 antibody (Fig. 2 *c*) could be completely blocked by preincubating the antibodies with 1 μ g of purified and heat-inactivated topoisomerase II α (Fig. 2 *d*), whereas the signal was not diminished by preincubation with heat-inactivated topoisomerase II β (Fig. 2 *e*). Similar fluorescent images of A431 cells were obtained with peptide antibodies recognizing a unique COOH-terminal epitope of topoisomerase II α . These could be blocked by preincubating the antibodies with 0.1 μ g of the immunogenic peptide or with 1 μ g of heat-inactivated recombinant topoisomerase II α but not with topoisomerase II β (not shown).

Topoisomerase II β was detected by peptide antibodies directed against the COOH terminus of the enzyme previously described in (6) and by the mouse monoclonal antibody 3H10. Fig. 1 attests to the ability of the peptide anti-

body (lane 14) and the monoclonal antibody 3H10 (lane 15) to react in Western blots with purified human topoisomerase II β and to stain uniformly a band of 180 kD in blots of whole A431 cell lysates (Fig. 1, lanes 16 and 17), similar in size to that of purified human topoisomerase II β detected by Coomassie blue staining (Fig. 1, lane 13). Cross-reactions with purified topoisomerase II α (not shown) or with topoisomerase II α present in the cell lysates (Fig. 1, lanes 16 and 17), could be excluded. Fluorescent images of A431 cells stained with monoclonal antibody 3H10 (Fig. 2 *f*) could be completely blocked by preincubating the antibody with 1 μ g of purified and heat-inactivated human topoisomerase II β (Fig. 2 *g*), whereas the signal was not diminished by preincubation with heat inactivated topoisomerase II α (Fig. 2 *h*). We also tested the specificity of the monoclonal antibody 8F8 used in previous studies for characterizing the cellular localization of topoisomerase II β (36, 68, 69). We observed that 8F8 antibody does not bind to Western blots of recombinant human topoisomerase II β purified from *S. cerevisiae*. Moreover, the nucleolar

staining of 8F8 antibody could not be blocked by preabsorption with purified human topoisomerase II β . The Western blot signal obtained with the 8F8 antibody in whole cell lysates had a different size (150 kD) than that obtained with peptide antibodies directed against a COOH-terminal epitope of human topoisomerase II β (180 kD; 6). Finally, the intensity of the 150-kD band stained by 8F8 antibody did not correlate to the level of topoisomerase II β -specific mRNA when several cell lines with different expression levels were compared, whereas the 180-kD band stained by the peptide antibody showed a clear correlation (63). We assume that these differences in immune-reactivity between 8F8 and the antibodies used in this study for labeling topoisomerase II β may explain some of the discrepancies between this and previous studies.

In summary, the data shown in Figs. 1 and 2 prove that we obtained selective and specific visualization of topoisomerases I, II α , and II β by Western blot and indirect immunofluorescence microscopy. Subsequently, we used these techniques for investigating the subcellular localization of topoisomerases in human epidermoid A431 cells.

Cellular Localization of Topoisomerases

Fluorescent images of A431 cells in logarithmic growth stained with antibodies specific for topoisomerases I (Fig. 3 *a*), II α (Fig. 3 *b*), or II β (Fig. 3 *c*) were bright, clearly different from each other, and also different from the DNA-patterns obtained by simultaneous staining with bisbenzimidazole (Hoechst 33258) which is shown for comparison. The overviews shown in Fig. 3 are representative for the whole cell population. Fig. 4 shows enlarged images of representative cells in interphase (4, *a-c*) or mitosis (4, *d-f*). Fig. 5 relates localization of topoisomerases to the nucleoli of interphase cells, whereas Fig. 6 shows localization of topoisomerases in isolated mitotic chromosomes.

Localization in Interphase. During interphase, the bulk of all three topoisomerases was localized in the nucleoplasm, whereas the cytosol was not significantly stained by any of the antibodies. Topoisomerase I mostly showed a diffuse, homogeneous distribution in the nucleoplasm throughout interphase (Fig. 3 *a*). In S phase, at least 30% of the cells exhibited spotted patterns (Fig. 4 *a*, top cell), and 10% showed bright, intranucleolar patches (Fig. 4 *a*, bottom cell). Intranucleolar localization of topoisomerase I was most prominent in S phase cells, but also detectable in G₁ and G₂ phase (not shown). Both type II topoisomerases showed a highly nonhomogeneous distribution in the nucleoplasm, which did not significantly change from G₁ through G₂ phase. Fig. 3, *b* and *c* shows representative examples of nonsynchronized cells in logarithmic growth. Topoisomerase II α appeared to be concentrated in numerous spots located extranucleolarly in the nucleus (Fig. 4 *b*, top cell). In addition, a less intensive homogeneous background staining of the nucleoplasm was observed, which was more pronounced inside nucleoli (Fig. 4 *b*, bottom cell). Topoisomerase II β exhibited a patchy reticular distribution, markedly different from topoisomerase II α . It was most dense in peri-nucleolar regions, but it was clearly always excluded from the interior of the nucleoli (Fig. 4 *c*). This finding, which is in contrast to previous reports (42, 68, 69), can be more clearly seen in the pseudo-

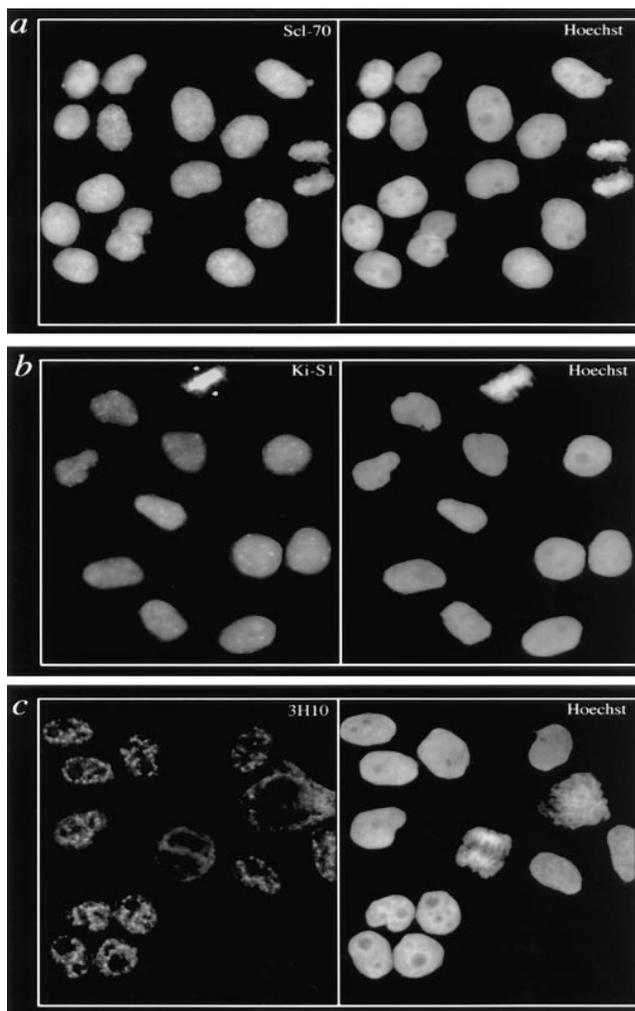


Figure 3. Fluorescent images of topoisomerases in human A431 cells. Monolayers of A431 cells grown on microscopic slides were fixed, permeabilized, and doublestained with bisbenzimidazole (Hoechst; right) and topoisomerase antibodies (left). (a) Immunostaining of topoisomerase I with Scl-70 autoantibodies. (b) Immunostaining of topoisomerase II α with Ki-S1 mouse monoclonal antibody. (c) Immunostaining of topoisomerase II β with 3H10 mouse monoclonal antibody.

colored overlay picture of DNA and immunofluorescent images shown in Fig. 5. Nucleoli could be discriminated as spherical areas within the chromatin, which were not stained by Hoechst (Fig. 5, *a-c*, left, arrows). In the Hoechst-negative intranucleolar space, immunostaining of topoisomerase II β was also negative. Consequently, in the stacked picture (Fig. 5 *c*, right), all of the red topoisomerase II β -specific signal (Fig. 5 *c*, middle) turns violet, due to colocalization with the blue Hoechst signal (Fig. 5 *c*, left). In contrast, stacked pictures of topoisomerase II α and DNA (Fig. 5 *b*, right) or topoisomerase I and DNA (Fig. 5 *a*, right) show prominent red immunosignals inside the Hoechst-negative nucleolar regions, which are not shifted to blue and can be distinguished from the violet signals obtained for enzymes colocalized with DNA in the adjacent chromatin. It can also be seen that topoisomerase II α exhibited a homogeneous intranucleolar staining only

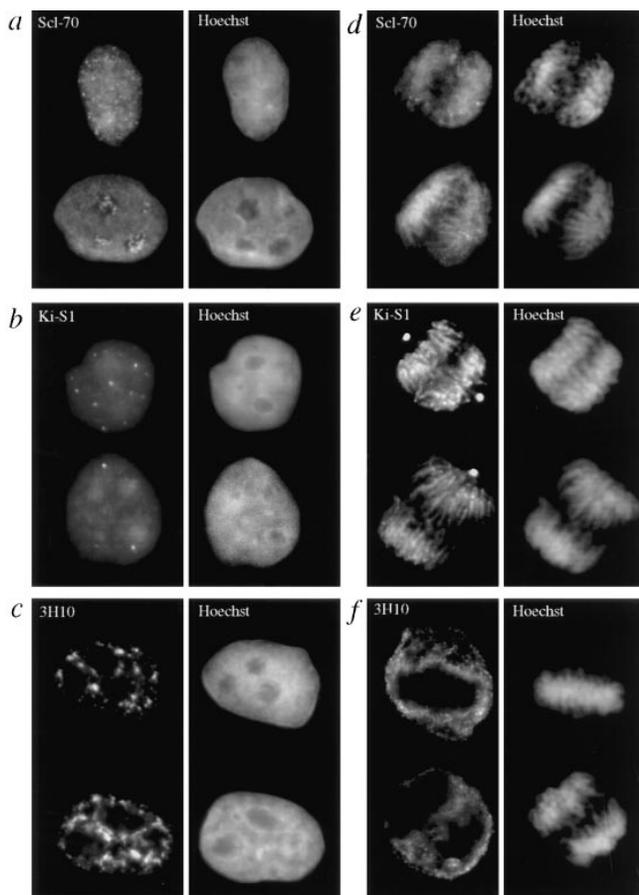


Figure 4. Localization of topoisomerases in interphase and mitosis. Close-up pictures of representative cells in interphase (*a–c*) or mitosis (*d–f*) immunostained for topoisomerase I (*a* and *d*), topoisomerase II α (*b* and *e*), or topoisomerase II β (*c* and *f*). The left of each pair of images represents immunostaining. The right shows the corresponding DNA pattern (Hoechst).

slightly more prominent than in the surrounding nucleoplasm, whereas topoisomerase I showed very prominent granular patterns inside the nucleoli, which are clearly brighter than the signal in the surrounding chromatin.

Localization in Mitotic Cells and Isolated Chromosomes. During mitosis, all of topoisomerase I remained attached to mitotic chromosomes. It showed a diffuse distribution in the chromosomal fibers (Fig. 4 *d*). Topoisomerase II α was also completely bound to the mitotic chromatin (Fig. 4 *e*), but in contrast to topoisomerase I it appeared to be highly concentrated in threadlike structures along the chromosome arms and in addition in the centriols (Fig. 4 *e* and Fig. 3 *b*). Topoisomerase II β diffused completely into the cytosol and was not detectable at all in the condensed chromatin (Fig. 4 *f*). Immunostaining of isolated chromosomes (Fig. 6) confirmed these observations. Topoisomerase I showed a diffuse, grainy pattern and was localized in the whole chromosomal fibers (Fig. 6 *a*), whereas topoisomerase II α was localized in numerous spots along the longitudinal axes of the chromosome arms (Fig. 6 *b*). Isolated chromosomes were not stained at all by topoisomerase II β antibodies (Fig. 6 *c*), confirming that during mitosis the bulk of the enzyme is not attached to the chromatin.

Nuclear Enzyme Levels and Specific Activities

Our observation that during mitosis topoisomerase II β was not bound to the DNA but diffused into the cytosol could either indicate that (*a*) the enzyme becomes inactivated, or (*b*) its activity remains unaltered, but binding to the condensed chromatin is inhibited sterically. Activity might also be modulated during mitosis in such a way that the equilibrium between bound and free enzyme becomes shifted towards the free form. Investigating these possibilities, we first compared the ability of all three topoisomerases to enzymatically interact with genomic DNA during mitosis: We treated A431 cells in logarithmic growth containing <1% mitoses and cultures arrested in metaphase containing >90% mitoses (see Table I) with camptothecin or etoposide, which stabilize the covalent complexes of DNA and topoisomerase I or II, respectively. Drug-induced covalent DNA linkage of catalytically active enzymes was monitored by immuno-band depletion, i.e., the inability of the covalently DNA-linked enzymes to enter SDS-polyacrylamide gels, resulting in a loss of the respective protein band in Western blots of whole cell lysates. Drug-induced band depletion was quantified by comparative densitometry of the immuno-blots (Table I). As shown in Fig. 7, a complete immuno-band depletion of topoisomerase II β was similarly observed in log-phase A431 cells and in cultures arrested in metaphase, indicating that all topoisomerase II β molecules, although mainly located in the cytosol, are capable of interacting with the condensed metaphase chromatin. In contrast, we observed that 15% of topoisomerase I and 50% of topoisomerase II α could not be linked to the chromatin of mitotic cells by camptothecin or etoposide, respectively, whereas a complete band depletion was obtained for both enzymes in log-phase cells (Fig. 7 and Table I). This might indicate that during mitosis, either the specific activity of these enzymes is largely reduced or a certain fraction of the enzymes is inactive or only partially capable of interacting enzymatically with the DNA.

To differentiate between these possibilities, we compared the amounts of topoisomerase antigens present in the nuclei of interphase and metaphase cells with the extractable catalytic activities. The results are summarized in Table I. Antigen levels of all three topoisomerases were higher (2.2-fold for topoisomerase I, 1.5-fold for topoisomerase II α , and 2.0-fold for topoisomerase II β) in cells locked in metaphase than in log phase cells, which is due to an increase in enzyme expression in S, G₂, and M phase, as shown in Fig. 8. These data are in close agreement with previous findings (2, 17, 28, 65). However, when measuring topoisomerase enzyme activities extractable from the nuclei, we obtained an unexpected result (Table I): in metaphase cells, activity of topoisomerase I extractable from each nucleus was reduced 2.2-fold, as compared to interphase cells. This decrease could be due to a lesser extractability of topoisomerase I during mitosis. We investigated this possibility by digesting the nuclear remnant with DNase after salt extraction, applying it to Western blotting, and comparing it to an equivalent number of non-extracted nuclei. As shown in Fig. 9 *a*, topoisomerase I antigen was extracted by >90% both from inter- and metaphase nuclei. Thus, the metaphase-associated decrease in

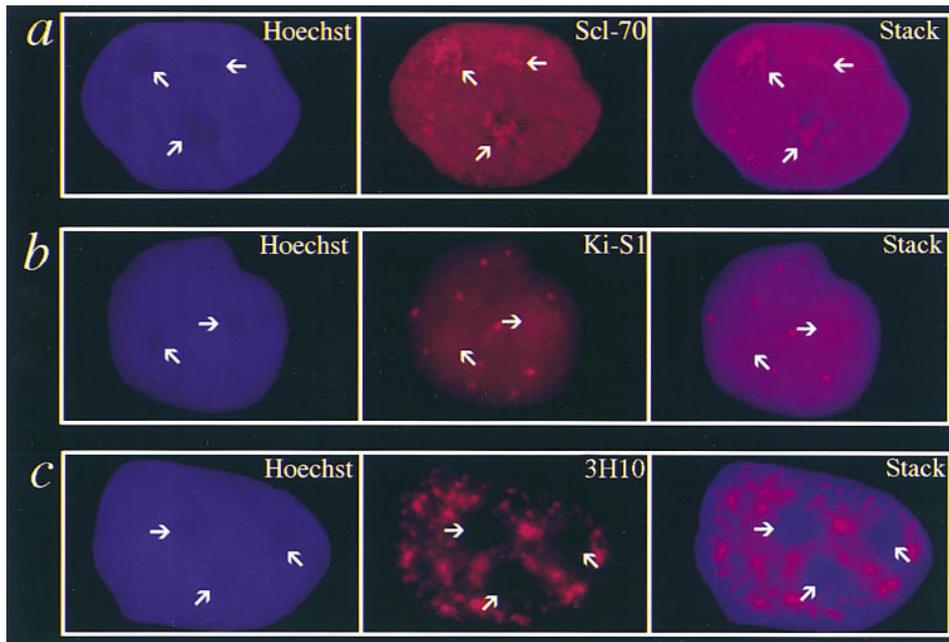


Figure 5. Colocalization of topoisomerases with DNA in interphase cells. Pseudo-color coded fluorescent images of topoisomerases (red) were stacked with corresponding patterns of bisbenzimid-stained DNA (blue). (Middle) immunostaining of topoisomerase I with Scl-70 (a), topoisomerase II α with Ki-S1(b), and topoisomerase II β with 3H10 (c). (Left) Corresponding image of bisbenzimid-stained DNA (Hoechst). (Right) Stacked image of immunostaining (red) and DNA (blue). Arrows indicate the positions of nucleoli.

topoisomerase I activity can not be explained by differences in extractability, but must be due to a decrease in specific catalytic activity. We calculated a 4.7-fold reduction of specific topoisomerase I activity in mitotic cells, taking into account that mitotic cells contain 2.2-fold more topoisomerase I antigen than interphase cells (Fig. 8). The significant decrease in specific catalytic activity may account for the slightly reduced drug sensitivity of topoisomerase I during mitosis (Fig. 7).

Topoisomerase II activity extractable from metaphase cells was reduced 1.7-fold as compared to interphase. Western blot analysis of the nuclear remainder digested with DNase after extraction showed that extractability of

topoisomerase II α was almost complete (88%) in interphase cells (Fig. 9 a, lanes 5 and 6), whereas in metaphase cells a substantial fraction (48%) escaped extraction (Fig. 9 a, lanes 7 and 8). In contrast, extractability of topoisomerase II β was >95% in interphase and in metaphase (Fig. 8 a, lanes 9–12). As we were not able to determine the individual contributions of α - and β -isoenzymes to overall topoisomerase II activity in the extracts, we can not decide whether the decrease in extractable topoisomerase II activity is solely due to reduced extractability of the α -isoenzyme or, in addition, to a decrease in specific activity of topoisomerase II β and/or of the extractable fraction of topoisomerase II α . However, it seems reasonable to as-

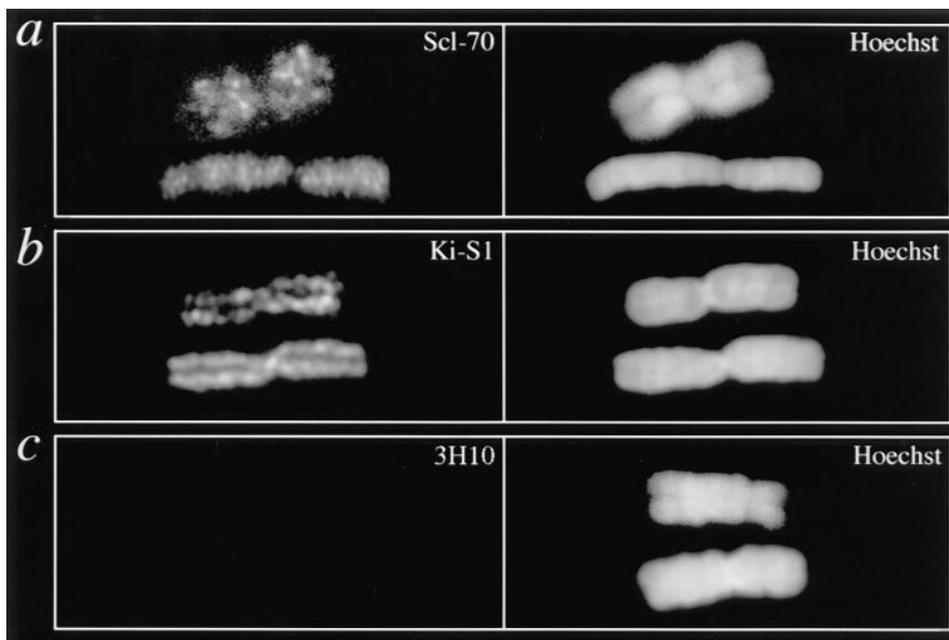


Figure 6. Localization of topoisomerases in isolated chromosomes. Isolated chromosomes were doublestained for DNA and topoisomerases I (a), II α (b), or II β (c), as in Fig. 3. Paired images of immunofluorescence (left) and corresponding DNA pattern (right) are shown.

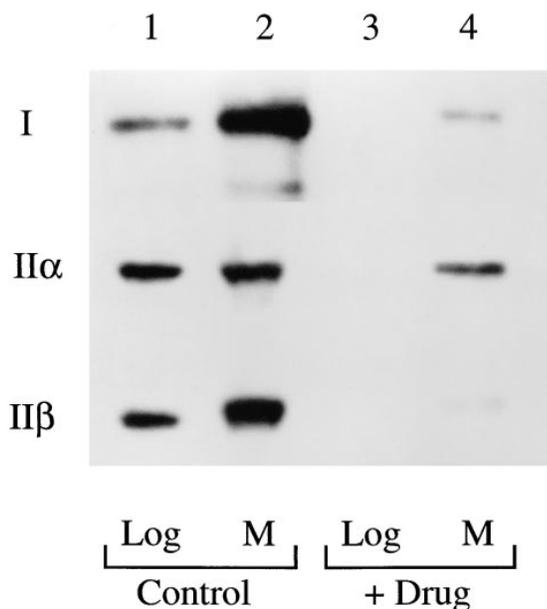


Figure 7. Drug-induced topoisomerase band-depletion in mitosis and interphase. Cells in logarithmic growth (*Log*) or blocked in metaphase by demecolcine (*M*) were treated with 30 μ M camptothecin (line 1, +*Drug*) or 200 μ M etoposide (lines 2 and 3, +*Drug*) for 1 h at 37°C. Controls were incubated without drug. Subsequently, the cells were harvested and lysed with hot SDS, and lysate equivalent of 5×10^5 cells was loaded onto each lane. Western blots were probed with rabbit peptide antibodies directed against the COOH termini of human topoisomerase I (*top*), II α (*middle*), or II β (*bottom*). This is a representative example of at least three identical experiments with similar outcome. A quantitative analysis of band intensities is given in Table I. The observed differences in band intensity between treated and untreated cells were significant on the 0.01 level (Wilcoxon's signed rank test).

sume that the dissociation of topoisomerase II β from the mitotic chromosomes is associated with an altered catalytic activity of the enzyme.

Noncatalytic Role of Topoisomerase II α in Mitotic Cells

As shown in Figs. 4 *e* and 6 *b*, the whole cellular complement of topoisomerase II α becomes concentrated in the long axes of the condensed chromosomes. In this situation, 50% of the enzyme molecules are not capable of interacting with the DNA in a catalytic manner, as can be deduced from their reduced drug sensitivity shown in Fig. 7. Drug insensitivity of topoisomerase II α in metaphase cannot be due to a reduced accessibility of topoisomerase II cleavage sites in the condensed chromatin, because the β -isoenzyme under the same conditions is capable of getting completely DNA linked in the presence of etoposide (Fig. 7). Thus, it must be due to a decrease in the catalytic activity of topoisomerase II α or physical separation of the active site of the enzyme from the DNA.

As cells proceed from S phase to mitosis, topoisomerase II α gets increasingly recruited to the nuclear scaffold fraction not extractable by 350 mM NaCl, whereas topoisomerase I and II β remain extractable by >90% throughout the cell cycle (Fig. 9 *b*). In metaphase, at least 48% of

Table I. Activity of Topoisomerases in Mitotic and Interphase A431 Cells. Mean Results of Three Independent Experiments

	Log culture	Metaphase*
<i>Cell cycle distribution</i>		
G ₁ , % [‡]	40	0
S, % [‡]	43	0
G ₂ /M, % [‡]	17	100
Mitotic index, % [‡]	< 1	96
<i>Topoisomerase I</i>		
pg/nucleus	0.1	0.22
Molecules/nucleus	6.2×10^5	13.8×10^5
U/nucleus [¶]	2.8×10^{-3}	1.3×10^{-3}
U/mg**	28×10^6	6.9×10^6
Band depletion by 30 μ M camptothecin, % ^{‡‡}	100	85
Extractability, % ^{§§}	98	96
<i>Topoisomerase IIα</i>		
fr/nucleus	13	20
Molecules/nucleus	4.7×10^4	7.3×10^4
Band depletion by 200 μ M etoposide, % ^{‡‡}	100	50
Extractability, % ^{§§}	88	52
<i>Topoisomerase IIβ</i>		
fg/nucleus	15	30
Molecules/nucleus	5.2×10^4	10.4×10^4
Band depletion by 200 μ M etoposide, % ^{‡‡}	100	98
Extractability, % ^{§§}	98	97
<i>Topoisomerase IIα + β</i>		
U/nucleus	8.3×10^{-5}	4.8×10^{-5}
U/mg ^{¶¶}	2.96×10^6	1.2×10^6

Standard errors of the mean were <20%.

*Cultured with 0.26 μ M demecolcine for 16 h.

[‡]Flow cytometry of bisbenzimidazole stained cells.

[§]Scored by fluorescence microscopy of bisbenzimidazole stained cells.

^{||}Comparative densitometry of immunoblots with purified enzyme as standard.

[¶]pBR 322 relaxation activity in the presence of orthovanadate.

^{**}Activity related to extractable amount of topoisomerase I antigen.

^{‡‡}Comparative densitometry. Density of immunoreactive enzyme band obtained in untreated cells was set to 0% and background value of identical position in an empty lane to 100%.

^{§§}Density of immunoreactive band obtained with whole nuclei was compared to that obtained with nuclear remnant after extraction with 350 mM NaCl and DNase digestion.

^{||}P4 DNA unknotting activity.

^{¶¶}Activity related to extractable antigen amount of both isoenzymes of topoisomerase II.

topoisomerase II α can not be extracted by 350 mM NaCl (Fig. 9, *a* and *b* and Table I). When comparing the drug sensitivity (i.e., the ability to covalently bind to DNA in the presence of etoposide) of extractable and nonextractable fractions of topoisomerase II α in metaphase cells (Fig. 8 *c*), it becomes apparent that actually the nonextractable portion is the one that does not interact with the DNA in a catalytic manner and consequently is refractory to etoposide treatment, whereas most of the extractable enzyme in the presence of etoposide (and ATP) forms a covalent complex with exogenously added calf thymus DNA, which is electrophoretically less mobile than the free enzyme (Fig. 9 *c*).

Taken together, these observations strongly support the notion that in mitotic chromosomes, at least 48% of topoisomerase II α serves a role not involving catalytic DNA turnover. Furthermore, in mitotic cells, topoisomerase II α is not only located in the chromosomal scaffold but also in the centriols (Figs. 3 *b* and 4 *e*). This observation implies

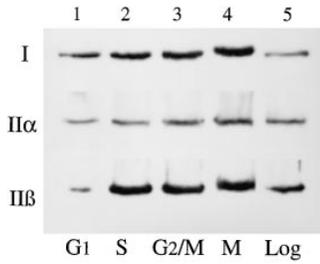


Figure 8. Cell cycle-coupled expression of topoisomerases. Cells were synchronized by demecolcine treatment and harvested at the indicated cell cycle stages (G_1 , S, G_2/M). Mitotic cells (M) were harvested directly after treatment with demecolcine for 16 h. Log-phase cells not treated with demecolcine (Log) served as controls. 5×10^5 nuclei isolated from the cells were lysed with hot SDS and applied to each lane. Western blots were probed with rabbit anti-peptide antibodies directed against the COOH termini of human topoisomerases I (*top*), II α (*middle*), or II β (*bottom*), respectively. This is a representative example of at least three identical experiments with similar outcomes. A quantitative analysis of band intensities is given in Table I.

that the enzyme might be one of the as yet unidentified components of the centrosome. As centrosomes of quiescent cells are not stained by topoisomerase II α antibodies (28, 49) and only one pair of centriols/centrosomes is stained in mitotic cells, it appears likely that the enzyme could play a functional role in the replication cycle and/or the maturation of the centriols (30).

Altered Phosphorylation of Topoisomerases during Mitosis

In mitotic cells, topoisomerase I and II β exhibited an in-

crease in apparent molecular weight (Fig. 8, lane 4), which has previously been assigned to M phase specific phosphorylation of these enzymes (10, 25, 26). When desalting nuclear extracts thoroughly by sequential precipitation with trichloroacetic acid and acetone and separating them in 5.5% polyacrylamide gels, which allow enhanced resolution in the 100–300-kD region, we observed that topoisomerase II α also exhibited a mitotic increase in apparent molecular weight. As shown in Fig. 10 (compare lanes 1 and 3), during mitosis, topoisomerase I was shifted by 2 kD from 102 to 104 kD. Topoisomerase II α was shifted from 170 to 175 kD, whereas topoisomerase II β showed a much larger shift (by 10 kD) from 180 to 190 kD. When extensively treating the extracts with alkaline phosphatase prior to electrophoresis, smaller and sharper immunoreactive bands were created in all cases (Fig. 10, lanes 2 and 4). For topoisomerase I, phosphatase treatment gave rise to a band of 101 kD, which was identical in interphase and mitotic cells and slightly smaller than the normal band position in interphase cells not treated with phosphatase (102 kD). Topoisomerase II α was shifted upon dephosphorylation to a band of 168 kD, which was similar in size in interphase and mitotic cells and significantly smaller than the normal band position of interphase cells not treated with phosphatase (170 kD). In the case of topoisomerase II β , phosphatase treatment in interphase decreased the size by 2 kD (from 180 to 178 kD), whereas in mitotic extracts it decreased by 8 kD (from 190 to 182 kD). However, even after extensive phosphatase treatment (50 U, 120 min, 37°C) there remained a size difference of 4 kD

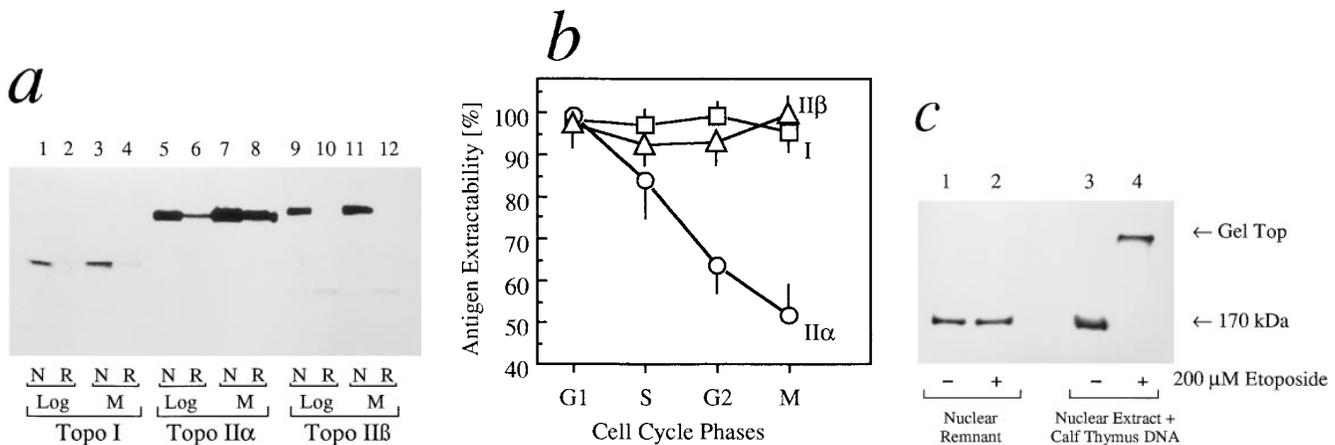


Figure 9. Cell cycle-coupled changes in DNA extractability of topoisomerases. (a) Nuclei were isolated from cells in logarithmic growth (Log) or blocked in metaphase by demecolcine (M). Nuclei were either lysed in hot SDS (N) or were first extracted with 350 mM NaCl, and the nuclear remnant (R) was lysed in hot SDS after digestion with DNase I (50 U/10⁶ nuclei) for 20 min at 37°C. 7×10^5 nuclei (N) or an equivalent amount of DNase-digested nuclear remnant (R) was applied to Western blotting. Blots were probed with rabbit peptide antibodies directed against the COOH terminus of human topoisomerase I (lanes 1–4), II α (lanes 5–8), or II β (lanes 9–12). This is a representative example of three identical experiments with similar outcomes. (b) The procedure described in a was performed with synchronized cells harvested in G_1 , S, or G_2 phase and with cells blocked in metaphase by demecolcine (M). The relative amounts of topoisomerases I, II α , and II β that were nonextractable by 350 mM NaCl as compared to total nuclear content, were determined by comparative videodensitometry of the immunoblots. Mean values of three identical experiments are plotted. Bars represent standard errors of the mean. (c) Nuclei were isolated from cells blocked in metaphase by demecolcine and extracted with 350 mM NaCl. Nonextractable nuclear remnants were dissolved in an equal volume of 3.5-fold diluted extraction buffer and incubated with (lane 2) and without (lane 1) 200 μ M etoposide in the presence of 1 mM ATP. Extracts were diluted 3.5-fold and incubated with (lane 4) and without (lane 3) 200 μ M etoposide in the presence of 1 mM ATP and 4 μ g calf thymus DNA. After 30 min the reaction was stopped by addition of hot SDS. Samples were sheared with a syringe and applied to Western blotting, and blots were probed with peptide antibodies directed against the COOH terminus of human topoisomerase II α . A representative example of three identical experiments with similar outcomes is shown.

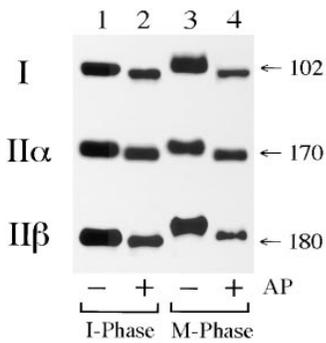


Figure 10. Treatment of nuclear extracts with alkaline phosphatase. Nuclei of mitotic (lanes 3 and 4) and interphase cells (lanes 1 and 2) were extracted with 800 mM NaCl. Extracts were precipitated with 3 M ammonium sulfate, renatured with diethanolamine buffer, pH 9.8, and incubated with 30 U of alkaline phosphatase at 37°C for 90 min (lanes 2 and 4). Controls (lanes 1 and 3) were incubated with an equivalent amount of phosphatase storage buffer without enzyme. Extracts were subsequently precipitated with 15% trichloroacetic acid. Precipitates were washed with acetone, dissolved in SDS sample buffer containing 5 M urea, separated on 5.5% polyacrylamide gels with a 3% stacking portion, transferred to Immobilon P membranes, and probed with peptide antibodies specific for the COOH-terminal portions of topoisomerase I (row 1), II α (row 2), or II β (row 3). Molecular weight values of enzyme specific protein bands indicated on the right margin were derived from Rf-plots of migration distances of marker proteins (see Materials and Methods) run in the same gel. The coefficient of variance of these molecular weight values was <20% in a run of three similar experiments, of which this one is a representative example. The observed differences in apparent molecular weight were significant on the 0.05 level (Wilcoxon's signed rank test).

incubated with an equivalent amount of phosphatase storage buffer without enzyme. Extracts were subsequently precipitated with 15% trichloroacetic acid. Precipitates were washed with acetone, dissolved in SDS sample buffer containing 5 M urea, separated on 5.5% polyacrylamide gels with a 3% stacking portion, transferred to Immobilon P membranes, and probed with peptide antibodies specific for the COOH-terminal portions of topoisomerase I (row 1), II α (row 2), or II β (row 3). Molecular weight values of enzyme specific protein bands indicated on the right margin were derived from Rf-plots of migration distances of marker proteins (see Materials and Methods) run in the same gel. The coefficient of variance of these molecular weight values was <20% in a run of three similar experiments, of which this one is a representative example. The observed differences in apparent molecular weight were significant on the 0.05 level (Wilcoxon's signed rank test).

between extracts of interphase and mitotic cells (Fig. 10, lanes 2 and 4). These data indicate that all three topoisomerases are phosphorylated during interphase as well as during mitosis, since phosphatase treatment induces a mobility shift in both cell cycle phases. However, during mitosis, phosphatase-induced mobility shifts are three to four-fold larger than in interphase, indicating that the enzymes are phosphorylated more or in a different way during mitosis. In the case of topoisomerase I and II α , the mitotic mobility shifts can be solely explained by an altered phosphorylation, since phosphatase treatment gives rise to bands of similar size in mitosis and interphase. In the case of topoisomerase II β , phosphatase treatment did not produce bands of similar size in mitosis and interphase. We concluded, that in addition to a phosphorylation-related increase in apparent molecular weight, there appears to exist a phosphatase-resistant shift of 4 kD, which could either be due to an unusual (phosphatase-resistant) type of phosphorylation of topoisomerase II β not present on the other topoisomerases, or, more likely, due to the presence of an additional posttranslational modification, which we have not yet been able to identify.

Discussion

In this study we present data on the localization and activity of type I and II DNA topoisomerases in human A431 epidermoid cells during mitosis and interphase. The cell line A431 was chosen for the investigation because it expresses unusually high levels of human topoisomerases, can be synchronized, and allows a clear morphological characterization of nuclei and cytoplasm after fixation. A431 cells are hypertriploid, which may in part explain their

overexpression of topoisomerases. Moreover, these cells do not stop logarithmic growth or undergo differentiation in postconfluent culture. Thus, our data are representative for cells in cell cycle but not necessarily for quiescent cells. The cell line was chosen because we wanted to avoid mixed effects of cell cycle arrest and differentiation induction, which are to be expected in cells able to induce differentiation genes upon growth arrest. Despite these restrictions, our results fit into a pattern emerging from accumulated knowledge about the general role of DNA topoisomerases in chromosome condensation and chromatin disjunction during the cell cycle (44), although some of our results conflict with those previously published on the cellular localization of DNA topoisomerases in other mammalian tumor cells (42, 68, 69).

Nucleolar DNA Topoisomerases

We found that topoisomerase II β is excluded from nucleoli during all stages of the cell cycle. This finding is in striking controversy to previous immunohistochemical studies, where the enzyme was mainly found inside the nucleoli (42, 68, 69). The intranuclear localization of topoisomerase II β and the increased expression during S phase (42; Fig. 8) and cellular differentiation (64, 65) have led to the hypothesis that topoisomerase II β mainly has a function in the transcription process of the heavily transcribed rRNA genes. However, this hypothesis is not supported by any direct biochemical evidence. In contrast, intranucleolar localization of topoisomerase I seen here and also reported before (33, 34, 36, 38) is supported by multiple biochemical observations implying a functional role of the enzyme in rRNA metabolism, such as preferred cleavage in regions flanking rRNA genes (8, 34), interaction with nucleolin (3), and phosphorylation of rRNA-splicing factors (46). Studies in yeast suggest that both type I and type II topoisomerases are required for the folding of rDNA into the functional organization of nucleolar genes, as cofactors of RNA polymerase I, and for the topological organization of the nucleolar chromatin (21). Our data suggest that in rapidly proliferating mammalian cells, which contain two isoforms of type II topoisomerases, this function is reserved to the α -isoenzyme which appeared to accumulate inside nucleoli of most interphase cells, whereas the β -isoenzyme was completely excluded from the nucleoli throughout interphase. We observed similar patterns in HeLa cells and stimulated human peripheral lymphocytes, whereas in primary cultures of nondividing cells, which do not express the α -form of topoisomerase II, topoisomerase II β appeared to be mainly localized in the nucleoplasm but also in the nucleoli. Most recently, Turley et al. (57) performed an extensive immunohistochemical analysis of the distribution of the two isoforms of DNA-topoisomerase II in various normal and neoplastic human tissues also using the 3H10 monoclonal antibody for visualizing topoisomerase II β . They showed that normal, quiescent tissues expressed only topoisomerase II β , which was localized in the nucleoplasm and the nucleoli. Expression of topoisomerase II α was restricted to the proliferative compartment of normal tissues and to tumors, where it exhibited a nucleolarly enhanced localization. From these observations it can be hypothesized that topoisomerase II α has a higher affinity to

the nucleolar chromatin than topoisomerase II β and that the extranucleolar localization of topoisomerase II β in A431 cells observed here could be due to the high levels of topoisomerase II α expressed in these cells (Table I), which may compete with topoisomerase II β for binding to the nucleolar chromatin. Thus, extranucleolar localization of topoisomerase II β should be a feature of rapidly proliferating cells expressing high levels of topoisomerase II α . It could be imagined that the differences between this study and previous studies on postconfluent Chinese hamster fibroblasts (42) reporting localization of topoisomerase II β in the nucleoplasm and in the nucleoli are due to the fact that A431 cells are a more pure model for rapidly cycling cells than postconfluent Chinese hamster fibroblast. However, reports on an exclusively nucleolar localization of topoisomerase II β in rapidly proliferating HeLa cells (68, 69) are clearly in contrast to our findings and all other available studies and are most likely due to differences in the specificity of the immunostaining methods. We believe that the antibodies and fixation procedures used here allow a more specific and selective labeling of topoisomerase II β in situ than those used in these other studies. Having purified recombinant human topoisomerases available in sufficient amounts, we could support our data by appropriate controls of specificity and isoenzyme selectivity of the immunological techniques used. Moreover, we could monitor localization of type I and II topoisomerases through the complete cell cycle and even in isolated mitotic chromosomes.

Localization and Activity of Topoisomerases in Mitotic Cells

A number of studies show that topoisomerase II is an abundant protein of the chromosomal scaffold which links the bases of the radial chromosomal DNA loops to the central protein core of the chromosomal fiber (12–14). In extracted or expanded human chromosomes, topoisomerase II antibodies identify a series of foci along the center of the long axis of the chromatid arms (13, 53). Similar data were obtained in indian muntiac cells (48). Until now, localization of α - and β -isoenzymes in mitotic chromosomes has not been studied directly. Thus, it was not completely clear to which of the two isoenzymes the structural role is assigned. The observation that activity of the α -isoenzyme is selectively inhibited by AT-rich oligonucleotides (11) suggested it as a candidate for binding to the chain of AT-rich sequences delineating the core of the chromatid fibers (48). Moreover, immunoprecipitates of the MPM-2 antibody directed against a phosphoepitope of mitotic scaffold proteins contained mainly the α -form of topoisomerase II (53). However, from studies in insect cells, which have only one form of topoisomerase II, it became apparent that only 30% of the enzyme is tightly linked to the core of the chromosome, whereas the rest diffuses away during mitosis (52). The concept of distinct populations of topoisomerase II, which either serve structural functions in the nuclear scaffold or are active in transcribed regions of the chromatin in a diffusible manner, is supported by the finding that in vivo, there exist two main classes of cleavage sites mapping either to scaffold adhesion sequences or to sites of transcriptionally active chromatin (44). The data

presented here indicate that at least in human A431 cells, two populations of topoisomerase II can be discriminated morphologically and can indeed be assigned to the two isoforms α and β . We show that the α -isoenzyme is tightly linked to mitotic chromosomes and exhibits a threadlike pattern along the long axis of the chromosome arms. It does not notably diffuse into the cytosol during mitosis. Moreover, as the cells progress from S phase to mitosis, topoisomerase II α gets increasingly recruited to the salt-stable scaffold protein fraction (Fig. 9, *a* and *b*), whereas the β -isoenzyme remains completely extractable through the whole cell cycle. In agreement with these observations, it has recently been reported that in mitotic human HeLa cells, topoisomerase II β is extracted by much lower salt concentrations than topoisomerase II α (26). Taken together, these observations strongly suggest that the α -isoenzyme is essential in chromosome condensation and disjunction, whereas the β -isoenzyme plays a minor role. This notion is also supported by preliminary results, showing that topoisomerase II β does not complement topoisomerase II α in NIH3T3 fibroblasts, expressing topoisomerase II α -antisense RNA (Andoh, T., S. Toji, and M. Kaneko. 1995. The Sixth Conference on Topoisomerases in Therapy: 28a.). The data shown in Fig. 9 *c* suggest that the salt-stable fraction of topoisomerase II (i.e., of the α -isoenzyme) in majority does not catalytically interact with the surrounding heterochromatin, as it is not getting DNA-linked in significant quantities, when exposed to high concentrations of etoposide (Fig. 9 *c*). In contrast, diffusible fractions of topoisomerase II (i.e., the extractable fraction of topoisomerase II α and topoisomerase II β) are getting completely DNA linked when exposed to etoposide. Thus, it appears that the diffusible and not the scaffold-linked topoisomerase II is the major drug target. These observations seem to be in contradiction to recent results by Gromova et al., demonstrating that treatment of salt-extracted nuclei with topoisomerase II poisons resulted in a similar pattern of long-range cleavage of genomic DNA cleavage as treatment of nonextracted nuclei (18). However, it should be noted that judging drug-induced covalent DNA linkage of topoisomerase II by immuno-band depletion, as done here, is insensitive to rare interactions involving <10% of the enzyme molecules, whereas measuring drug-induced DNA-cleavage, as done by Gromova et al., (18) does not indicate how large a fraction of the topoisomerase II molecules is involved in the process. Taken together, our results and the data of Gromova et al. indicate that a small fraction of scaffold-bound topoisomerase II α is catalytically active and consequently targeted by topoisomerase II poisons, whereas the majority is catalytically inactive.

Our observation that topoisomerase II α localizes to the centrioles of dividing cells provides a novel finding, further indicating that functions of topoisomerase II α extend beyond DNA turnover. It has been shown that centrioles replicate in a semiconservative manner, independent of DNA and protein synthesis (41). Interestingly, in quiescent cells, centrosomes are not stained by topoisomerase II α specific antibodies (28, 49), indicating that the localization of topoisomerase II α to the centrioles is restricted to cycling cells. The functional role of topoisomerase II α in the replication cycle of centrioles remains to be established.

In interphase cells, topoisomerase II α appears to be

highly condensed in various confined areas located within the nucleoplasm, but outside the nucleoli. These spots were more frequent in late S and G₂ phase. Moreover, we previously observed formation of similar clusters of topoisomerase II α in the nuclei of peripheral human lymphocytes upon stimulation with phytohemagglutinin 2 (28). It has been suggested that multimerization of topoisomerase II α could play a role in formation of the nuclear scaffold of mitotic chromosomes (58). Thus, it might be imagined that the clusters observed here actually represent such multimers of topoisomerase II α forming at defined organization centers within the euchromatin of interphase cells, from which condensation of chromosomes originates, as the cell prepares to enter mitosis. In this context, it is interesting to note that recently it has been shown that a fraction of topoisomerase II α clusters at centromeric regions of the chromatin as cells prepare to enter mitosis and the enzyme is required for proper centromere/kinetochore structure (45).

The majority of topoisomerase II β clearly does not have a structural role in mitotic chromosomes as it diffuses away from the heterochromatin. This notion is supported by the observation that salt extractability of the β -isoenzyme does not change during the cell cycle. The data shown in Table I suggest that diffusional loss from the heterochromatin could be at least partially due to a decrease in catalytic activity, which might shift the DNA-binding equilibrium of the enzyme towards the unbound form. However, all of the enzyme molecules remain active and capable of catalytically interacting with the DNA during mitosis, as shown by their undiminished susceptibility to etoposide. This is in clear contrast to the partial refractoriness of the α -isoenzyme. Recently, it has been shown that topoisomerase II α and β can form low amounts of heterodimers *in vivo*, as well in yeast as in human HeLa cells (4). Here we observed a more or less complete spatial dissociation of topoisomerase II α and β antigens in mitotic A431 cells, which seems to argue against the existence of significant levels of heterodimers in these cells. The discrepancy could be due to the fact that heterodimers were present in too small amounts to be detected. Heterodimers might also be specific for interphase cells and not present during mitosis. In agreement with our data, it has previously been observed that immunoprecipitates of mitotic scaffold phosphoproteins of mouse P388D1 lymphocytic cells obtained with the MPM-2 antibody contained only traces of topoisomerase II β , which were shown not to be due to α/β -heterodimerization (53).

Topoisomerase I, like topoisomerase II α , remains attached to mitotic chromosomes and does not notably diffuse into the cytosol. It has been reported before that topoisomerase I is enriched at the centromeres before anaphase (33). However, the mitotic pattern and the immunostaining of isolated chromosomes shown here (Figs. 4 *d* and 6 *a*) rather argue in favor of a diffuse distribution in the periphery of the chromosomes, which is in clear contrast to the thread-like pattern of topoisomerase II α . Since salt extractability of topoisomerase I did not change in mitosis as significantly as that of topoisomerase II α (Fig. 9 *b*), it seems unlikely that the type I enzyme has a structural role in chromosomes similar to that of topoisomerase II α .

In agreement with previous publications (10, 25, 47), we observed expressional upregulation of all three topoisom-

erases during the cell cycle. The enzymes were phosphorylated during interphase as well as mitosis but during mitosis phosphorylation appeared to be enhanced or altered, as could be deduced from a significant increase in phosphatase-sensitive electrophoretic retardation of the enzyme bands in SDS-polyacrylamide gels (Fig. 10). A large body of evidence suggests that phosphorylation increases catalytic activity of topoisomerase I as well as topoisomerase II *in vitro* (9, 19). Thus, we were puzzled by our observation that specific activity of topoisomerase I and II decreased in metaphase cells, although the enzymes were apparently more phosphorylated than in interphase cells. It is widely assumed that phosphorylation of topoisomerases *in vivo* has the same stimulatory effect as phosphorylation *in vitro* and that consequently topoisomerases should be most active during mitosis where phosphate incorporation is highest. However, previous comparisons of topoisomerase activities in nuclear extract from S phase and mitotic cells did ambiguously support this concept (15, 55). Moreover, it has been observed in cells resistant to etoposide, that hyperphosphorylation can inactivate topoisomerase II (54) suggesting that *in vivo* regulation of topoisomerase activity by phosphorylation might actually be biphasic, stimulating enzyme activity during S and G₂ phase but inhibiting it in mitosis due to phosphorylation of additional or novel sites. On the other hand, it might be that during mitosis, catalytic activity of topoisomerases is modulated by additional factors, such as β -glycerophosphate, a component of mitotic extracts, which *in vitro* has a marked inhibitory effect on the enzymatic activity of topoisomerase II and stimulates its multimerization (58). The data shown in Fig. 10 give some indication of an additional covalent post-translational modification of topoisomerase II β during mitosis, which increases the apparent molecular size of the enzyme by 3.8 kD in a phosphatase-insensitive fashion. We do not yet know what causes this phosphatase-insensitive size shift. However, topoisomerase II β is exported to the cytosol during mitosis. In A431 cells, enzyme levels drop significantly from M to G₁ phase (Fig. 8). This seems to indicate a rapid postmitotic degradation of topoisomerase II β , although in HeLa cells the protein moiety of topoisomerase II β appears not to be degraded as the cells go from mitosis to G₁ phase (26). In light of recent findings emphasizing the important role of protein ubiquitination in the degradation of cell cycle related proteins at the end of mitosis (22, 35), it is tempting to speculate that the mitotic export of topoisomerases II β to the cytosol, and possibly also its subsequent degradation, might be triggered by ubiquitination or some other posttranslational modification independent of phosphorylation.

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