Topoisomerase II: A Specific Marker for Cell Proliferation

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Abstract. We have used an antibody probe to measure the levels of topoisomerase II in several transformed and developmentally regulated normal cell types. Transformed cells contain roughly 1×10^6 copies of the enzyme. During erythropoiesis in chicken embryos the enzyme level drops from 7.8×10^4 copies per erythroblast to <300 copies per erythrocyte concomitant with the cessation of mitosis in the blood. Cultured myoblasts also lose topoisomerase II upon fusion into nonproliferating myotubes. When peripheral blood lymphocytes (which lack detectable topoisomerase II) commence proliferation, they express topoisomerase II de novo. Appearance of the enzyme exactly parallels the onset of DNA replication. These results suggest that topoisomerase II is not required for transcription in higher eukaryotes, but that it may function during DNA replication. Furthermore, topoisomerase II is a sensitive and specific marker for proliferating cells.

TOPOISOMERASES are enzymes that alter the topological state of DNA. These enzymes relax supercoiled DNA by transient protein-linked cleavages of either one (type I enzymes) or both (type II enzymes) DNA strands (31, 55). Type II topoisomerases also catenate and decatenate closed circular duplex DNA. The mechanisms of enzyme action have been studied extensively in vitro (31, 55). In contrast, the role of the eukaryotic enzymes in vivo has been amenable to analysis only recently, since the development of specific antibody probes (3, 12, 18) and the isolation of conditional lethal mutants in yeasts (6, 19, 53).

Type II topoisomerases have been implicated in several aspects of DNA metabolism and structure, including replication (26, 37), sister chromatid exchange (39, 45), transcription (22, 33), organization of chromosomal loop domains (4, 17), and chromosome disjunction at mitosis (6, 24, 35, 48, 49, 53, 54). In addition, topoisomerase II may be an important structural protein since it is a major component of mitotic chromosome scaffold (9, 12, 18) and interphase nuclear matrix (3) fractions. In mitotic chromosomes the enzyme is apparently concentrated at the base of the radial loops (9). This was the location predicted by the radial loop model of chromosome architecture for proteins involved in establishment and/or maintenance of loop structure (28).

Genetic studies in yeasts suggest that the major essential function of topoisomerase II is to allow chromosomal disjunction (6, 24, 53, 54). Topoisomerase II mutants remain viable at the restrictive temperature, provided that mitosis is blocked by inhibitors of microtubule polymerization (24). Thus, while topoisomerase II may be involved in other cellular processes in yeasts, topoisomerase I can evidently substitute to carry out these functions if necessary (6, 19, 53, 54).

In the course of our earlier studies, we observed that the levels of topoisomerase II (detected by indirect immunofluorescence) appeared to vary widely from cell to cell (12). Use of a fluorescence-activated cell sorter indicated that cells with reduced levels of the antigen consistently had a prereplicative (G_1) DNA content (12). However, only a small percentage of the G_1 cells was negative for topoisomerase II. This suggested that either the antigen might be degraded for a brief interval during G_1, or that the negative cells might no longer be dividing.

A possible link between topoisomerase II activity and cell proliferation has been suggested by studies in which enzyme activity is measured in proliferating and growth-arrested cells. Duguet et al. (7) showed that the low level of enzyme activity in liver increased significantly after partial hepatectomy. Tandou et al. (50) found a low level of enzyme activity in G_0 guinea pig lymphocytes that increased dramatically after mitogenic stimulation. A third study, using Chinese hamster ovary (CHO) cells recovering from serum starvation-induced G_0 arrest, found that drug-induced cleavable DNA complexes (thought to form in vivo due to inhibition of the topoisomerase II religation reaction) increased 4–6 h after the onset of DNA synthesis (47).

Unfortunately, activity assays are sensitive to factors, such as efficiency of enzyme solubilization and cell cycle variability in specific activity, that cannot be quantified without specific antibody. We therefore decided to rigorously test the hypothesis that topoisomerase II is a specific marker for proliferating cells by examining a number of systems in which the proliferative activity of defined populations of cells undergoes a programmed developmental change. In erythropoiesis and myogenesis, blast cells cease dividing concomitant with terminal differentiation. During lymphocyte activation, resting (G_0) cells are induced to divide in response to specific stimuli. In all three cases, the processes occur with a high degree of natural synchrony. The immunological assay that we have used to detect topoisomerase II provides an accurate measure of the total cellular level of accumulated polypeptide.

We found that topoisomerase II is an abundant protein

in both normal and transformed cells. The high levels of topoisomerase II were greatly reduced when cells ceased proliferation. This occurred not only in quiescent cells (erythrocytes), but in cells that remained transcriptionally active (myotubes). In contrast, circulating peripheral blood lymphocytes, which initially lacked detectable topoisomerase II, were found to express the antigen de novo following mitogenic stimulation in vitro. In these cells, levels of topoisomerase II rose coordinately with the onset of DNA replication, consistent with our earlier observation that cells lacking topoisomerase II had a prereplicative (G_1) DNA content. Our findings provide strong support for the concept that topoisomerase II is a specific marker for proliferating cells.

Materials and Methods

Buffers

The following buffers were used. D-PBS contained 8.06 mM Na_2HPO_4, 1.47 mM KH_2PO_4 (pH 7.5), 137 mM NaCl, and 2.7 mM KCl. Buffer A contained 15 mM Tris/HCl pH 7.4, 80 mM KCl, 2 mM K-EDTA, 0.5 mM spermidine, and 0.2 mM spermine plus 8.5% sucrose. Buffer 3 contained 5 mM Tris/HCl, pH 7.4, 2 mM KCl, 2 mM K-EDTA, and 0.25 mM spermidine plus 8.5% sucrose.

Immunoprecipitation and Quantitation of Topoisomerase II

Immunoadsorption and gel analysis was carried out essentially as described by Ottaviano and Gerace (38). Cell solubilization buffer also included 2 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg/ml each of chymostatin and antipain. In addition, aliquots (e.g., 2%) of the immunoprecipitate and resultant cell supernatant were analyzed by immunoblotting to determine the efficiency of immunoadsorption. The ratio of topoisomerase II in supernatant to pellet (which varied between 20 and 0.2) was used in the quantitation calculations.

Isolation and Processing of Avian Erythrocytes

Blood cells were collected from severed embryonic vessels into ice-cold D-PBS containing 1 mM EDTA, 1 mM PMSF, and 0.1% Trasylol. Blood was obtained from adult chickens by bleeding from the wing vein. Cells were washed three times by pelleting at 1,000 *g*. At this point, aliquots of cells were spun through PBS at 400 *g* onto concanavalin A–treated coverslips (14). Concanavalin A (Calbiochem-Behring Corp., Indianapolis, IN) was dissolved at 0.5 mg/ml in 150 mM NaCl, 10 mM Tris, pH 7.4, 0.5 mM $MnCl_2$, and 0.5 mM $CaCl_2$ to increase stability. Coverslips were processed for immunofluorescence as described (13) with the modification that, after permeabilization in 0.1% Triton X-100, KB (wash and antibody dilution buffer) contained 0.02% Triton X-100. After hemacytometer counting, cells were pelleted, sonicated, and boiled in SDS sample buffer (12).

Erythroid cell samples (typically 1–2 × 10^6 cells/lane) were electrophoresed in multiple 10% polyacrylamide gels (30). After transfer to nitrocellulose (51) in the presence of 0.1% SDS, blots were processed as described (10) with primary antibody and iodinated protein A. Quantitation was done by densitometric scanning of appropriate preflashed autoradiographic exposures. Alternatively, nitrocellulose blots were stained for 10 min in D-PBS containing 0.3% Tween-20 and 1 μl/ml India ink (21). Desired regions were then excised and quantitated in a gamma counter.

In Vitro Myogenesis

11- or 12-d chick embryo thighs were collected into warm DME and dissected free of bones and overlying skin. After gentle mincing, tissue clumps were dissociated in 0.05% trypsin and 0.25 mM EDTA for 30 min at 37°C with intermittent pipetting to facilitate dissociation. Trypsinization was stopped by the addition of horse serum to 10%, and cells were washed twice in DME + 10% horse serum + 5% embryo extract (27). Cells were plated onto gelatin coated glass coverslips at a density of 2 × 10^6 cells/60 mm dish, and the medium changed after 24 h at 37° in a humidified 5% CO_2 incubator. Immunofluorescence was performed as described above.

Autoradiographic Analysis of Transcription

4-d myotube cultures were incubated with 50 μCi/ml 5,6-[^{3}H]uridine (Amersham Corp., Arlington Heights, IL; 48 Ci/mmol) for 3 h. After a 20-min chase in medium without label, coverslips were processed for immunolabeling with the substitution of strepavidin: horse radish peroxidase conjugate (Bethesda Research Laboratories, Gaithersburg, MD) for the usual fluorescent streptavidin: Texas Red conjugate. Diaminobenzidine served as the reaction substrate. Coverslips were mounted cell side up on microscope slides with Krazy-glue and dipped in NTB-2 (Kodak) emulsion diluted 1:1 with H_2O. Slides were developed in D-19 (Kodak) developer. Exposure times were typically on the order of 4 d. To obtain bright field images that contained all the desired information (i.e., autoradiographic grains, topoisomerase II localization, and cytoplasmic visualization), slides were stained with 0.05% toluidine blue in 0.1% NaB_4O_7:10 H_2O for 20–30 s and rinsed in water. Images were photographed on an Olympus Vanox AH-2 microscope (S plan apo 60X N.A. 1.40 lens) on Kodak EL400 film with exposure times of 1–3 s.

Avian Lymphocyte Isolation and Culture

Lymphocytes were isolated from heparinized peripheral blood by a differential centrifugation method as described in Hovi et al. (25). White blood cells were cultured at a density of 5 × 10^6 cells/ml in RPMI 1640 (pH 7.4) containing 10 mM HEPES (pH 7.4), 5% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol. Mitogens (PHA-M form, Gibco, Grand Island, NY or concanavalin A, ICN, Plainview, NY) were added at time 0 at dilutions of 1:50 or 5 μg/ml, respectively (and were present throughout the experiment). Methyl-[^{3}H]thymidine (Amersham Corp.; 87 Ci/mmol) was added also at time 0 for replication studies at 0.5 or 1 μCi/ml. Incorporation of label into DNA was assayed by TCA precipitation. At appropriate time points, 0.4 ml of culture (2 × 10^6 cells) were pelleted in an Eppendorf microfuge and solubilized in SDS sample buffer. The presence of topoisomerase II was assayed by immunofluorescence (cells attached to concanavalin A–treated coverslips) and by immunoblotting of the protein gel samples.

Results

Measurement of Topoisomerase II Levels in Normal and Transformed Cells

We have determined the amount of total cellular topoisomerase II in two transformed cell lines (MSB-1 and line 249) and one normal cell population (5-d erythroblasts) by quantitative immunoprecipitation. Topoisomerase II immunoprecipitates from a known number of cells were subjected to SDS-PAGE. The amount of precipitated protein was determined by elution of the Coomassie Blue from excised gel slices followed by spectrophotometry (16). Absorbance measurements were converted to protein mass units by comparison with values obtained from known amounts of BSA electrophoresed in the same gel (see Materials and Methods).

The results of a typical experiment, in which total cellular proteins were solubilized and immunoprecipitated with anti-topoisomerase II, are shown in Fig. 1. A single precipitated polypeptide of 170 kD was observed in samples exposed to specific antibody, and was absent from controls exposed to preimmune serum. Quantitation of the amount of precipitated 170-kD polypeptide demonstrated that topoisomerase II is a very abundant protein in these cells (Table I). The two transformed lines contained ~10^6 copies of enzyme/cell (Table I). Chicken erythroblasts (5 d) contained 7.5 × 10^4 copies. Because these cells already show a 50% decrease in topoisomerase II level, as we show below, we calculate that the early proliferating erythroblasts have at least 1.5 × 10^5 copies of enzyme per cell. The higher values obtained for transformed cells may reflect the greater proliferative activ-

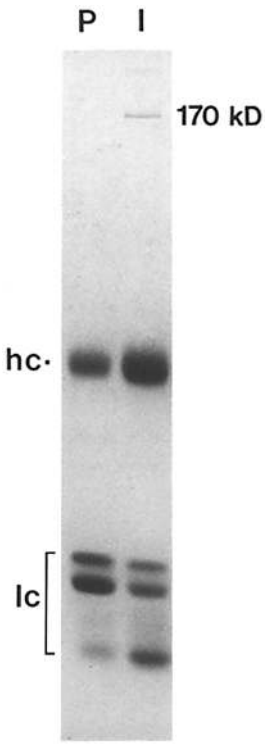

Figure 1. Immunoprecipitation of topoisomerase II from 5-d embryonic erythroid precursors. 2 μl of guinea pig preimmune (*P*) or immune (*I*) serum (12) was added to 5×10^7 solubilized cells. Immune complexes were sedimented after the addition of fixed *Staphylococcus aureus*, solubilized in SDS sample buffer, and electrophoresed in a 10% polyacrylamide gel. The gel was stained with Coomassie Blue. The immune precipitate contained only topoisomerase II (170 kD), and the heavy (*hc*) and light (*lc*) chains of the immunoglobulins.

ity of these cells compared with the nontransformed erythroblasts.

A similar Coomassie-binding method was independently used to estimate the amount of topoisomerase II in human (HeLa) mitotic chromosomes and chromosome scaffolds (18). In that study, total protein from chromosomes and scaffolds was subjected to SDS PAGE. The authors then used the dye-elution assay to determine the amount of a 170-kD polypeptide (inferred to be solely topoisomerase II). That study yielded a number (2.8×10^5/cell) slightly lower than those we obtained by immunoprecipitation. This difference could reflect either a lower level of the enzyme in HeLa cells or losses of antigen incurred during the isolation of chromosomes by Gasser et al. (18).

Erythropoiesis in Chicken Embryos

Erythropoiesis in the chicken embryo has been widely used for the study of the regulation of patterns of gene expression during differentiation. Cells of the embryonic mesenchyme give rise to "primitive" (first generation) proerythroblasts that divide and mature in the circulating blood (32, 56). Between 2 and 5 d, the circulating red cells are limited to this primitive cohort, which differentiates with natural synchrony (32, 42). At 3–4 d of embryogenesis, the blood contains a uniform population of primitive polychromatic erythroblasts (32). These cells are mitotically active in the blood for the first 8 d of development (42; this study, data not shown).

At later times (6–8 d), the situation is more complex, and mature primitive erythrocytes coexist in the blood with late "definitive" polychromatic erythroblasts. These populations are difficult to distinguish, as the nuclei of mature primitive erythrocytes lack the oblate shape and extensive heterochromatin that characterize the nuclei of mature definitive erythrocytes. From 8 d onward, these early generations are progressively superceded by the mature definitive erythrocytes, and the composition of the blood again becomes effectively uniform at 10 d of embryogenesis (42).

Table I. Numbers of Topoisomerase II Monomers per Cell in Several Cell Types

Cell type	Number of copies	Number of determinations
MSB-1 lymphoblastoid	$1.6 \pm 0.81 \times 10^6$	15
249 hepatoma	$7.6 \pm 3.4 \times 10^5$	3
5-d erythroblasts	$7.8 \pm 1.6 \times 10^4$	6

Values are derived from quantitation of the amount of 170-kD polypeptide that could be immunoprecipitated from a known number of cells (Materials and Methods).

Analysis of Erythroid Cells by Immunofluorescence

Blood was obtained from developing embryos at 3 d (and at intervals of 24 h thereafter) and red cells separated from contaminating yolk platelets by gentle centrifugation. An aliquot of the cells was processed for indirect immunofluorescence, and the remainder was boiled directly in SDS sample buffer for subsequent electrophoresis (see next section). To minimize possible proteolysis, all steps were performed in the presence of trasylol, PMSF, chymostatin, leupeptin, antipain, and pepstatin.

At 3-4 d development, virtually all (99%) primitive polychromatic erythroblasts contained levels of topoisomerase II readily detected by indirect immunofluorescence (Fig. 2, *a–c*). In erythroblasts, as in cultured cells (12), the antigen was localized in nuclei and mitotic chromosomes. Shortly thereafter, the percentage of immunopositive cells began to decline, and by 6 d development, only 53% of the cells contained detectable levels of antigen (Fig. 2, *d–f*).

The percentage of immunopositive cells dropped rapidly during subsequent development, reaching 2% at 10 d. From 10 to 18 d this percentage remained constant (Fig. 2, *g–i*). Immunopositive cells were independently recognizable under phase contrast, since they had larger, more euchromatic nuclei (Fig. 2 *h*). These are apparently rare circulating erythroblasts that persist late into development (H. Weintraub, personal communication). Blood from a chick (15 d post-hatching) or adult chicken contained no erythroid cells positive for topoisomerase II by immunofluorescence (not shown).

These results suggest that topoisomerase II disappears from erythroid cells concomitant with the loss of proliferative ability.

Detection of Topoisomerase II in Erythroid Cells by Immunoblotting

Since results obtained by immunofluorescence depend on accessibility of the antigens to the antibody, we also examined the cells for the presence of topoisomerase II by immunoblotting techniques. In each experiment, isolated blood cells were subjected to SDS PAGE in parallel gels. One gel was stained with Coomassie Blue (Fig. 3 *a*) and the others electrotransferred to nitrocellulose. In all cases, samples derived from an equal number of cells were loaded in each lane. Since a decrease in cell volume by ~70% accompanies the initial stages of erythroid maturation (42), the total amount of protein per lane decreased over the course of the experiment (Fig. 3 *a*).

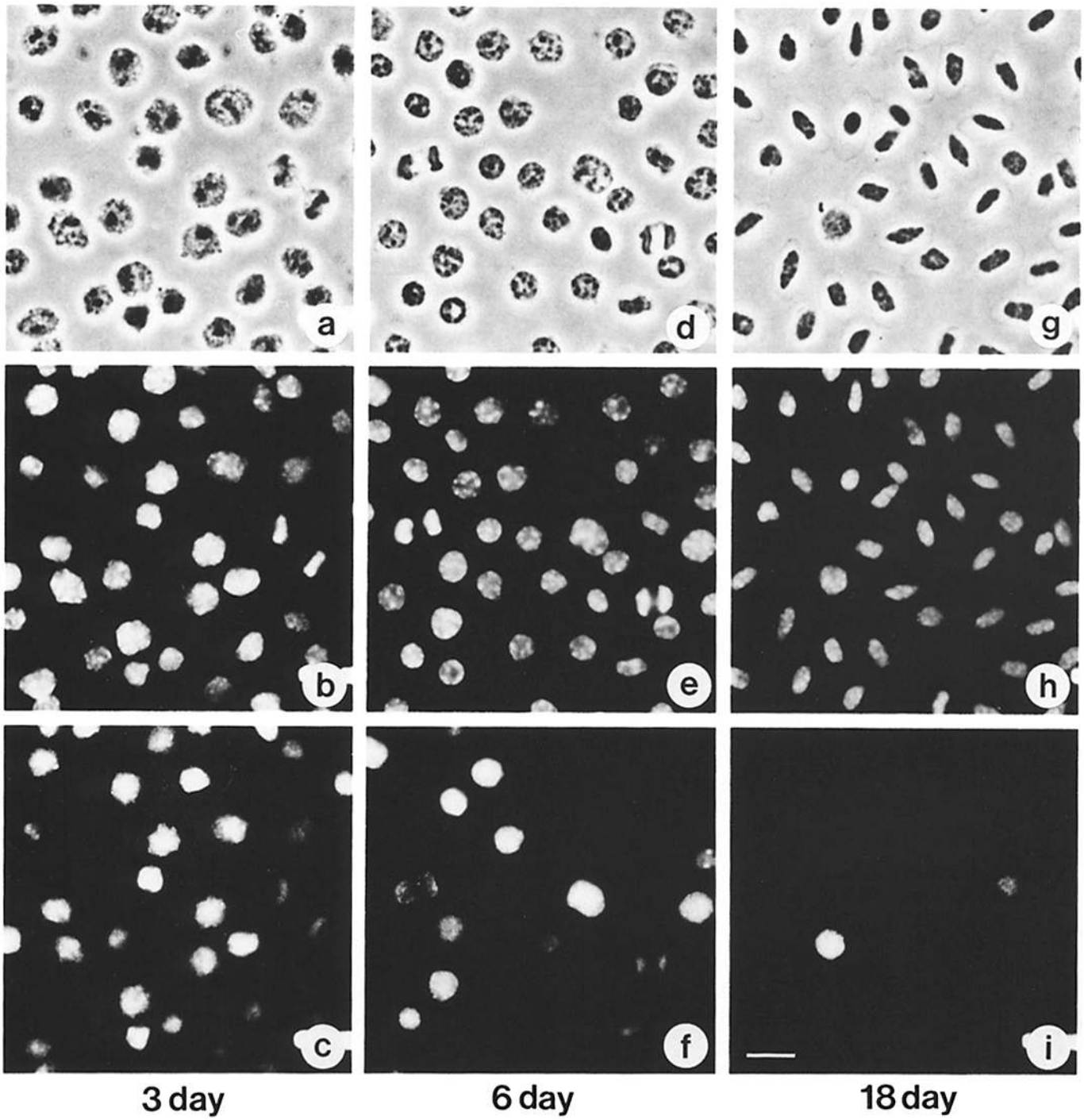

Figure 2. Immunofluorescent visualization of topoisomerase II in isolated chicken embryonic erythroid cells. Cells were attached by centrifugation onto concanavalin A-treated coverslips and processed for immunofluorescence. Embryonic age is indicated for each series of images beneath the panel. Phase contrast (*a*, *d*, and *g*), DAPI staining of DNA (*b*, *e*, and *h*), and topoisomerase II localization (*c*, *f*, and *i*). Bar, 10 μm.

Control blots were probed with a serum specific for erythrocyte β-tubulin (gift of Dr. Douglas Murphy, Johns Hopkins University) and with a human autoantibody recognizing topoisomerase I (44). The amounts of tubulin and topoisomerase I decreased during maturation by ~65% (Fig. 3 *b*), and 62% (Fig. 4), respectively. Therefore the ratio of both proteins to cell volume remained approximately constant.

A significantly different pattern was observed when similar nitrocellulose blots were probed with antiserum to topoisomerase II (Fig. 3 *c*). In this case, the antibody binding decreased dramatically over time. The data of Fig. 3 *c* were quantified by densitometry of multiple prefogged films exposed for appropriate times. The levels of topoisomerase II present in erythroid cells from day 3 onward are presented in Fig. 5. Approximately 50% of the initial binding was lost between days 4 and 5, and a basal level of 2% was reached by day 10. This low level of binding remained constant throughout the second half of embryogenesis and corre-

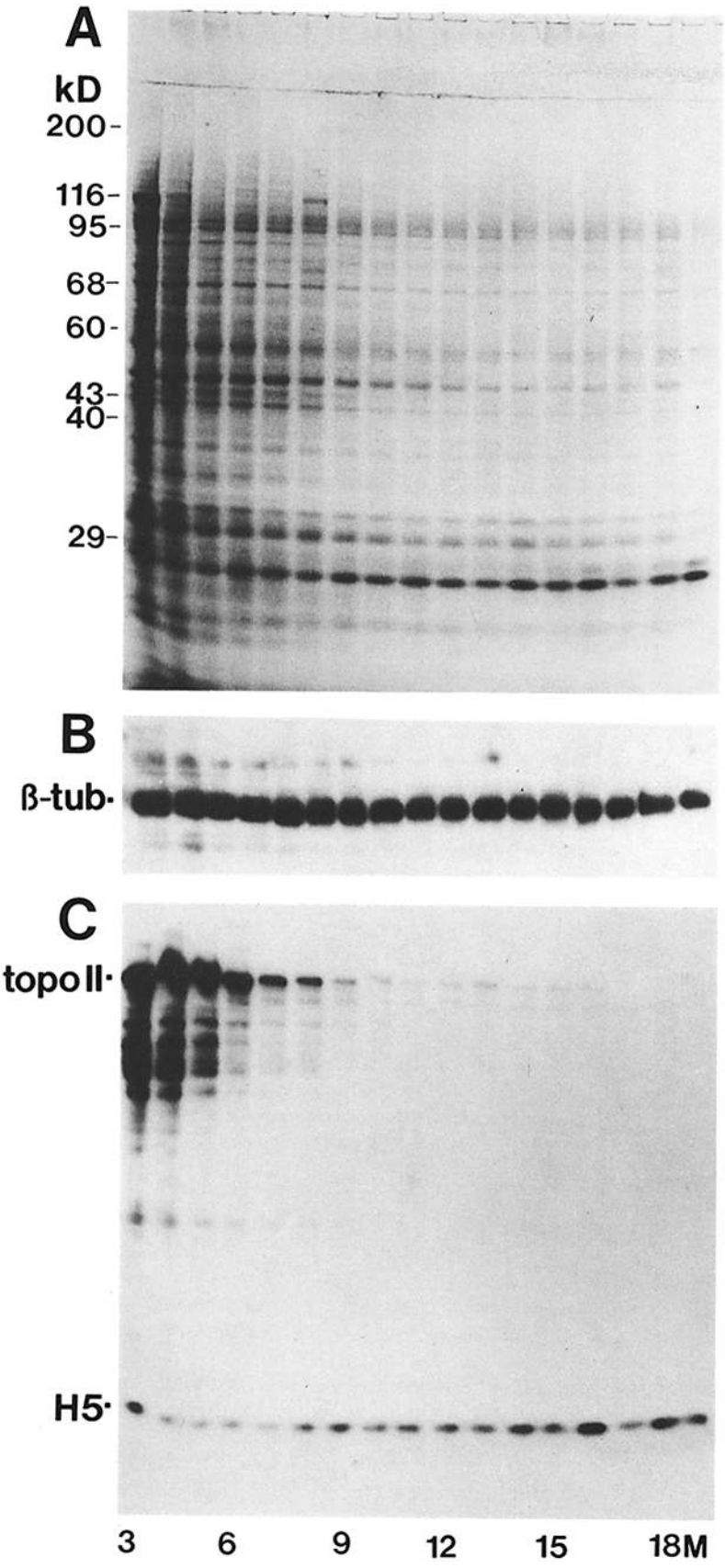

Figure 3. SDS PAGE and immunoblotting analysis of total extracts from embryonic and adult erythroid cells. Cells (1.25×10^6/lane) were washed in PBS containing protease inhibitors at 4°C, lysed by boiling and sonicating in sample buffer, and electrophoresed in 10% polyacrylamide gels. (*A*) Coomassie Blue-stained gel. (*B* and *C*) Nitrocellulose transfers from gels run in parallel probed with (*B*) an erythrocyte-specific antibody to β-tubulin and (*C*) anti-topoisomerase II. Bound antibody was visualized by autoradiography with ^{125}I-protein A. Embryonic age in days is indicated at the bottom. M is blood from an adult (mature) chicken. The mobilities of standard proteins (myosin, 200 kD; beta-galactosidase, 116 kD; phosphorylase b, 95 kD; bovine serum albumin, 68 kD; catalase, 60 kD; actin, 43 kD; aldolase, 40 kD; carbonic anhydrase, 29 kD) are indicated to the left of *A*.

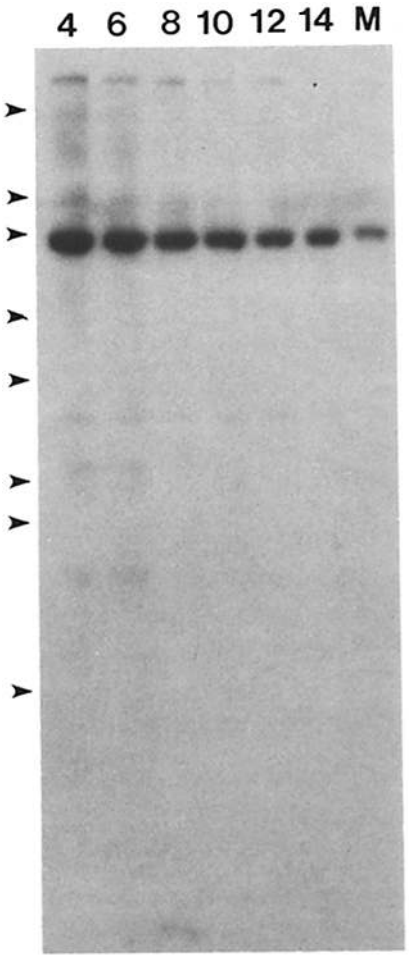

Figure 4. Detection of topoisomerase I in erythroid cells by immunoblotting. Total cell extracts were prepared and analyzed as in Fig. 2. The nitrocellulose blot was probed with a human autoimmune serum recognizing topoisomerase I. Numbers at the top indicate the embryonic age in days. *M*, adult chicken erythrocytes (1.9×10^6 cells were loaded per lane). Arrowheads indicate the position of the molecular weight standards (as in Fig. 2).

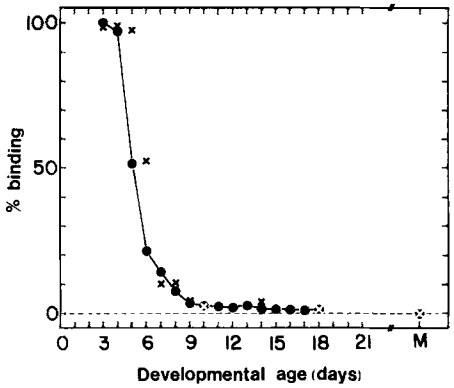

Figure 5. Quantitation of the loss of topoisomerase II during chicken erythropoiesis. Appropriate exposures of immunoblots like that of Fig. 3 were scanned with an integrating densitometer. The density of both cleaved and intact topoisomerase II were included. These values are represented as the percent of binding normalized to the 3-d value (*solid circles*). The percentage of immunopositive cells at each time point (measured by indirect immunofluorescence) is also designated (×). From 10 d onward, the level of embryonic topoisomerase II remains constant at 2% (equal to the percentage of immunopositive cells). Adult chicken erythrocytes (designated M at right of the figure) contain no topoisomerase II detectable by either immunofluorescence or immunoblotting.

sponded precisely to the percentage of cells containing topoisomerase II detectable by immunofluorescence (Fig. 2). This suggests that the antigen detected in immunoblots arose from the rare circulating erythroblasts.

We could not detect topoisomerase II in mature chicken erythrocytes, even in overexposed immunoblots from lanes loaded with 4×10^6 mature erythrocytes. In contrast, in parallel experiments we have detected topoisomerase II from as few as 750 MSB-1 cells. Given our estimation of 1.6×10^6 copies/MSB-1 cell, we conclude that the mature erythrocyte has an upper limit of 300 copies of the enzyme per cell.

Such conclusions from immunoblotting experiments could be influenced by two potentially serious concerns. First, the antitopoisomerase II antiserum (which was raised against the scaffold-associated form of the enzyme) might detect only a subfraction of the total topoisomerase II. We believe this to be unlikely, since an independent antiserum raised against purified topoisomerase II from bovine calf thymus (20) gave identical results in immunoblotting experiments (data not shown). Differences in cross-species immunoreactivity between these two antisera argue strongly that they recognize different epitopes on the protein. Second, mature red cells might contain proteases that degrade the topoisomerase II during preparation of the gel samples. We have attempted to minimize this possibility by use of a mixture of protease inhibitors during the cell isolation steps, and by disruption of the cells directly in boiling SDS sample buffer (Materials and Methods). Using this procedure, we did not detect immunoreactive proteolytic fragments of either topoisomerase I, β-tubulin, or histone H5 in immunoblots. Moreover, in additional experiments we have assayed for topoisomerase II-specific protease activity by mixing a constant number of MSB-1 cells with increasing numbers of 11-d erythrocytes (in ratios up to 1:125). When these mixtures were processed for immunoblotting as above, the total amount of topoisomerase II remained constant at all ratios tested. Taken together, these results suggest that the disappearance of topoisomerase II does not result from the presence of high levels of nonspecific proteases in the mature erythroid cells.

Both antitopoisomerase II antibodies showed significant binding to a protein band of ~29 kD (Fig. 3 *c*). Since it cofractionated with nuclei and was therefore a candidate for being a stable erythroid-specific breakdown product of topoisomerase II, the identity of this antigen was investigated further. The following four observations (data not shown) suggest that this antigen is not a proteolytic fragment of topoisomerase II, but rather is histone H5, and that the binding of antibodies to it is nonspecific. (*a*) The 29-kD polypeptide was excised from a preparative polyacrylamide gel and injected into two guinea pigs. Both animals produced antibodies that recognized the antigen in immunoblots. One of these antisera also stained the nuclei of erythroid cells at all stages of development in indirect immunofluorescence. (*b*) Neither antibody showed detectable binding to topoisomerase II in immunoblots. However both antibodies strongly recognized

two highly purified fractions of H5 (gift of R. D. Cole, University of California, Berkeley) in similar experiments. (*c*) Bona fide histone H5 was not immunoprecipitated by the topoisomerase II antibody. (*d*) We have previously observed artefactual histone binding by other antibodies against chromosomal nonhistone proteins using our immunoblotting protocol (10, 13). The degree of binding varies, depending on the protocols used for washing the blots, and is probably due to the highly positively charged nature of the histones. We conclude that the 29-kD polypeptide is most probably histone H5 and is unrelated to topoisomerase II.

Thus, detection of total cellular topoisomerase II by immunoblotting confirms that the antigen is lost from embryonic erythroid cells concomitant with the cessation of cell division.

Loss of Topoisomerase II during Myogenesis

We next wished to determine whether loss of topoisomerase II was correlated with the cessation of proliferation or with the progressive loss of transcriptional activity that occurs during erythropoiesis. Several indirect observations (22, 33) suggest that transcription might require torsional strain of the template, presumably due to action of a type II topoisomerase. This question has been approached by examining two cell types (myotubes formed by cell fusion in vitro and hepatocytes from adult liver). Although each of these cell types no longer divides, both retain a high degree of transcriptional activity.

For culturing myoblasts, single cell suspensions rich in myoblasts and fibroblasts were obtained from thighs of 11-d chicken embryos. When plated at a suitable density, myoblasts fused to form myotubes after ~3 d in culture. Nuclei in myotubes no longer divide, but continue to actively transcribe the genes required for muscle function (23). The assembly of sarcomeres in these myotubes was confirmed by indirect immunofluorescence with a monoclonal antibody to myosin (gift of Dr. Thomas D. Pollard, Johns Hopkins School of Medicine; data not shown). Myotube development proceeded normally under these conditions, since the syncytia eventually began to spontaneously contract, often resulting in their detachment from the culture dish.

When myoblast cultures were analyzed by immunofluorescence 2 d after plating, virtually all cells were immunopositive for topoisomerase II (Fig. 6, *a–c*). Fusion of myoblasts into myotubes was associated with loss of detectable topoisomerase II antigen. At 4 d postplating none of the myotube nuclei contained detectable topoisomerase II antigen, while many surrounding fibroblasts remained immunopositive (Fig. 6, *d–f*). In particular, dividing cells were consistently found to react strongly with the antibody. In no case were nuclei within syncytia found to contain detectable topoisomerase II.

To confirm that the myotube nuclei were transcriptionally active under our experimental conditions, we pulse-labeled fused cultures (4 d postplating) with [^{3}H]uridine and performed simultaneous immunolocalization and autoradiography. This was done by replacing the streptavidin/Texas Red conjugate used for indirect immunofluorescence with a streptavidin/horse radish peroxidase conjugate. To enhance the contrast of the cytoplasm, slides were also briefly stained with toluidine blue. The brown peroxidase reaction product and black silver grains were then photographed simultaneously under bright field illumination (Fig. 7). All fibroblasts exhibited transcriptional activity, but varied in topoisomerase II content, with a few negative cells distributed among the majority of positive cells. In all cases, the clustered nuclei of myotubes (marked *m*) were covered by silver grains indicative of transcriptional activity and did not contain detectable levels of topoisomerase II.

This finding, taken together with the observation that nuclei from hepatocytes were negative for topoisomerase II when assayed by both immunofluorescence and immunoblotting (data not shown), indicates that the reduction in amount of topoisomerase II that accompanies terminal differentiation has no apparent effect on the ability of cells to transcribe RNA.

Appearance of Topoisomerase II during Lymphocyte Activation

There is direct evidence that a type II topoisomerase is required for DNA replication in prokaryotes (26, 37). In addition, indirect evidence suggests that the enzyme might fulfill a similar role in eukaryotes (6, 47–50, 54). To test whether the stoichiometric amounts of topoisomerase II detected by our antibody are required for DNA synthesis in eukaryotic cells, we measured topoisomerase II levels after mitogenic activation of peripheral blood lymphocytes. This approach has advantages that (*a*) before stimulation the level of DNA synthesis is negligible, and (*b*) stimulated cells begin replicating as a relatively synchronous wave. Thus, it is possible to separate events of late G_1 and of mitosis from those of S phase.

It is well known that peripheral blood lymphocytes (in G_o phase) may be stimulated to enter the cell cycle in response to an immunological challenge in vivo or in response to a number of mitogens in vitro. Since phytohemagglutinin and concanavalin A are strict T cell mitogens, they were chosen for these studies in order to maximize the synchrony of the response.

Chicken peripheral blood lymphocytes, when first isolated, were found not to express topoisomerase II antigen in amounts detectable by immunofluorescence (Fig. 8, *a–c*) or immunoblotting. However, after 72 h in culture, antigen could be detected in 5–30% of the cells (Fig. 8, *d–f*). These results were confirmed by immunoblotting experiments (data not shown), which indicated that the appearance of the antigen reflected de novo protein synthesis in response to the onset of proliferation. While the efficiency of stimulation showed slight variability between experiments, the overall pattern of the results remained constant. Similar results were obtained with both mitogens.

To examine the temporal relationship between the appearance of topoisomerase II and the onset of DNA replication, activation was carried out in the presence of trace amounts of [^{3}H]thymidine. Incorporation of label into acid insoluble products was determined at appropriate intervals. At the same time, known numbers of cells were solubilized in SDS sample buffer for immunoblotting and aliquots were processed for indirect immunofluorescence.

In each experiment, aliquots were taken at 2-h intervals beginning at 20 h. Replication was first detected at ~28 h after plating. Aliquots taken at 0, 3, 6, 9, and 18 h after the onset of replication were processed for indirect immunofluorescence and immunoblotting. Though the immunoblot-

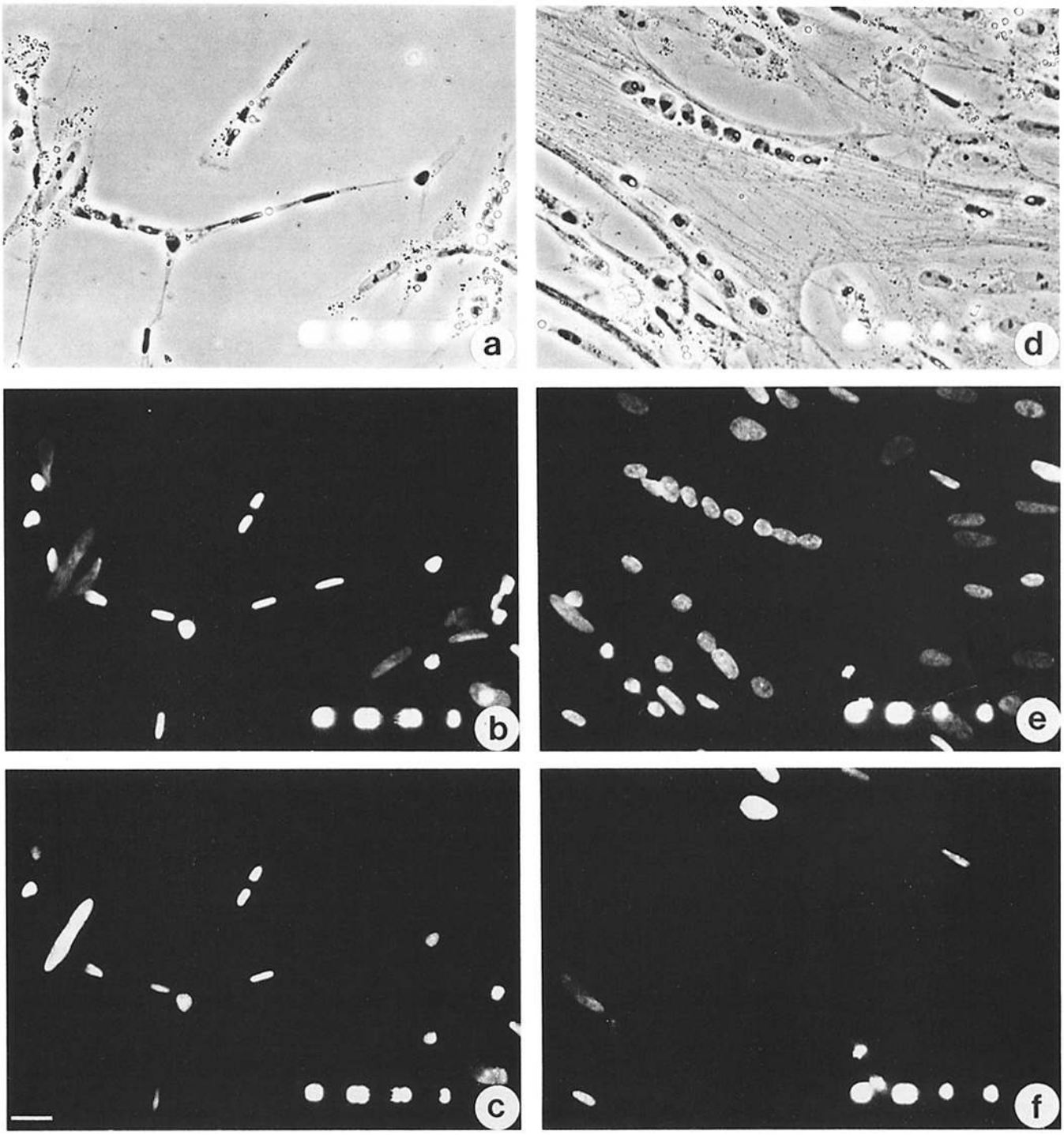

Figure 6. The fate of topoisomerase II during in vitro myogenesis as visualized by immunofluorescent staining with antitopoisomerase II. 2 d after plating (*a–c*), almost all myoblasts and fibroblasts contain topoisomerase II. 4-d cultures (*d–f*) contain extensive myotubes. Nuclei of myotubes lack detectable topoisomerase II. Phase-contrast images (*a*, *d*), DAPI staining of DNA (*b*, *e*) and immunofluorescent localization of topoisomerase II (*c*, *f*). Bar, 20 μm.

ting experiments provided a measure of the total accumulation of antigen in the culture, the level of antigen in individual cells is not known.

Few, if any, immunopositive cells were detected before the initiation of replication (Fig. 9). Instead, the percentage of immunopositive cells rose concomitant with the onset of DNA synthesis as did the appearance of antigen as detected by immunoblotting (not shown). Given the small percentage of cells that became detectably immunopositive, this emphasizes the sensitivity of the immunoblotting approach.

In other experiments, aliquots of cells taken 0, 2, 4 and 22 h after the onset of replication were processed for autoradiography and immunolocalization of topoisomerase II. These cells were pulsed with 5 μCi/ml [^{3}H]thymidine for 30 min before harvesting. Simultaneous detection of replication and antigen in individual cells confirmed the above

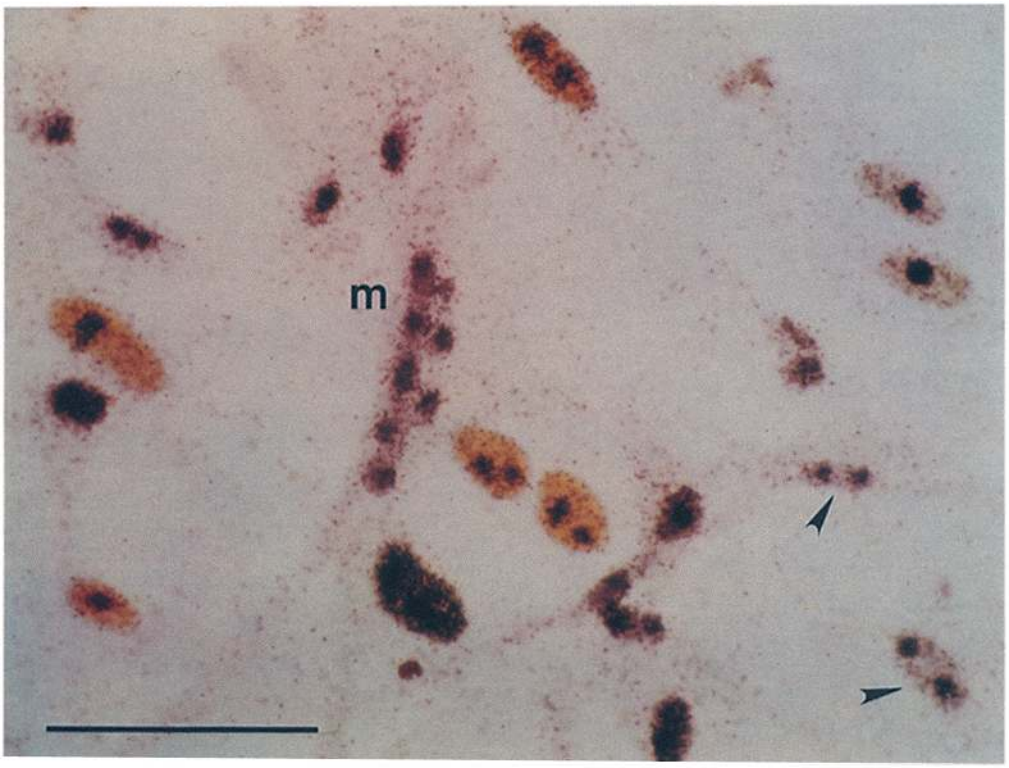

Figure 7. Demonstration of transcriptional activity in myotube nuclei lacking detectable topoisomerase II. *m*, myotube. All other cells are fibroblasts. Before fixation for immunolabeling by the peroxidase method (brown reaction product), cells were exposed to [^{3}H]uridine to detect transcriptional activity (3-h pulse, 20-min chase). Note that while all cells exhibit silver grains (black), topoisomerase II content varies. Myotube nuclei were always negative for the antigen. Two fibroblasts negative for topoisomerase II are indicated (*arrowheads*). All others show varying amounts of the antigen. Slides were briefly stained with toluidine blue to enhance cytoplasmic contrast. Bar, 50 μm.

results, that is, virtually all (295/300) S phase cells had detectable levels of topoisomerase II. No cells were observed in which topoisomerase II could be detected in the absence of DNA synthesis until very late times, when it was expected that many of the cells would have progressed into G_2 phase.

These results suggest that stoichiometric amounts of topoisomerase II do not accumulate late in G_1 before the initiation of replication and confirm our earlier observation that all S-phase cells contain detectable levels of the antigen.

Discussion

An important insight into the possible role(s) of topoisomerase II in higher eukaryotes came from the observation of Sundin and Varshavsky (48, 49) that the terminal stages of SV40 replication involve production of multiply intertwined catenated circles which are apparently resolved by a type II topoisomerase. They also suggested that the enzyme might perform a similar function in chromosome disjunction at mitosis (49). Recent analyses of mutants defective for topoisomerase II in *Saccharomyces* (6, 19, 24) and *Schizosaccharomyces* (53, 54), as well as a detailed analysis of the topoisomerase II (gyr B) mutants in *Escherichia coli* (46), support the initial hypothesis of Sundin and Varshavsky. Unfortunately, unambiguous analysis of yeast mutants has been complicated by the apparent ability of topoisomerases I and II to substitute for each other in vivo. This functional redundancy has prevented genetic dissection from clearly establishing the role of the enzymes in transcription and replication.

Genetic approaches in yeasts have been complemented by immunobiochemical studies in higher eukaryotes. Topoisomerase II has been shown to be a component of two highly insoluble protein fractions from chromosomes and nuclei: the mitotic chromosome scaffold (12, 18) and the interphase nuclear matrix (3). While it remains for future experiments to demonstrate whether the insolubility of these subcellular fractions in vitro arises from their being derived from preexisting structures in vivo, these observations suggest that topoisomerase II might be an integral structural component of the nucleus.

Recent observations suggesting that the genome of eukaryotes is constrained into topological domains containing roughly 100 kb (reviewed in reference 8) offer one possible structural role for topoisomerase II—i.e., the enzyme might be one of the nonhistone components responsible for topological closure of the domains. In a previous study, we showed that topoisomerase II is apparently located at the base of the radial loops of mitotic chromosomes (9). Evidence that the enzyme is similarly positioned in interphase nuclei comes from studies of specific DNA fragments that remain associated with the nuclear matrix/scaffold fraction using the method of Mirkovitch et al. (34). High resolution mapping studies indicate that such attached fragments are significantly enriched in topoisomerase II consensus cleavage sequences (4, 17, 43).

Using antibodies to measure total topoisomerase II polypeptide, we have shown that rapidly proliferating transformed cells contain $\sim 1 \times 10^6$ copies of the enzyme. This amount of enzyme is similar to the number of individual nuclear lamin polypeptides found in the peripheral lamina of rat liver nuclei (L. Gerace, personal communication). The levels of topoisomerase II measured by immunoprecipitation are thus consistent with the enzyme fulfilling a structural role in organizing the genome. Since the chicken genome is 2.4×10^9 bp (or $\sim 2.4 \times 10^4$ 100 kb loop domains), there are ~20 topoisomerase II dimers per domain (roughly one dimer per 25 nucleosomes). Alternatively, the interphase ge-

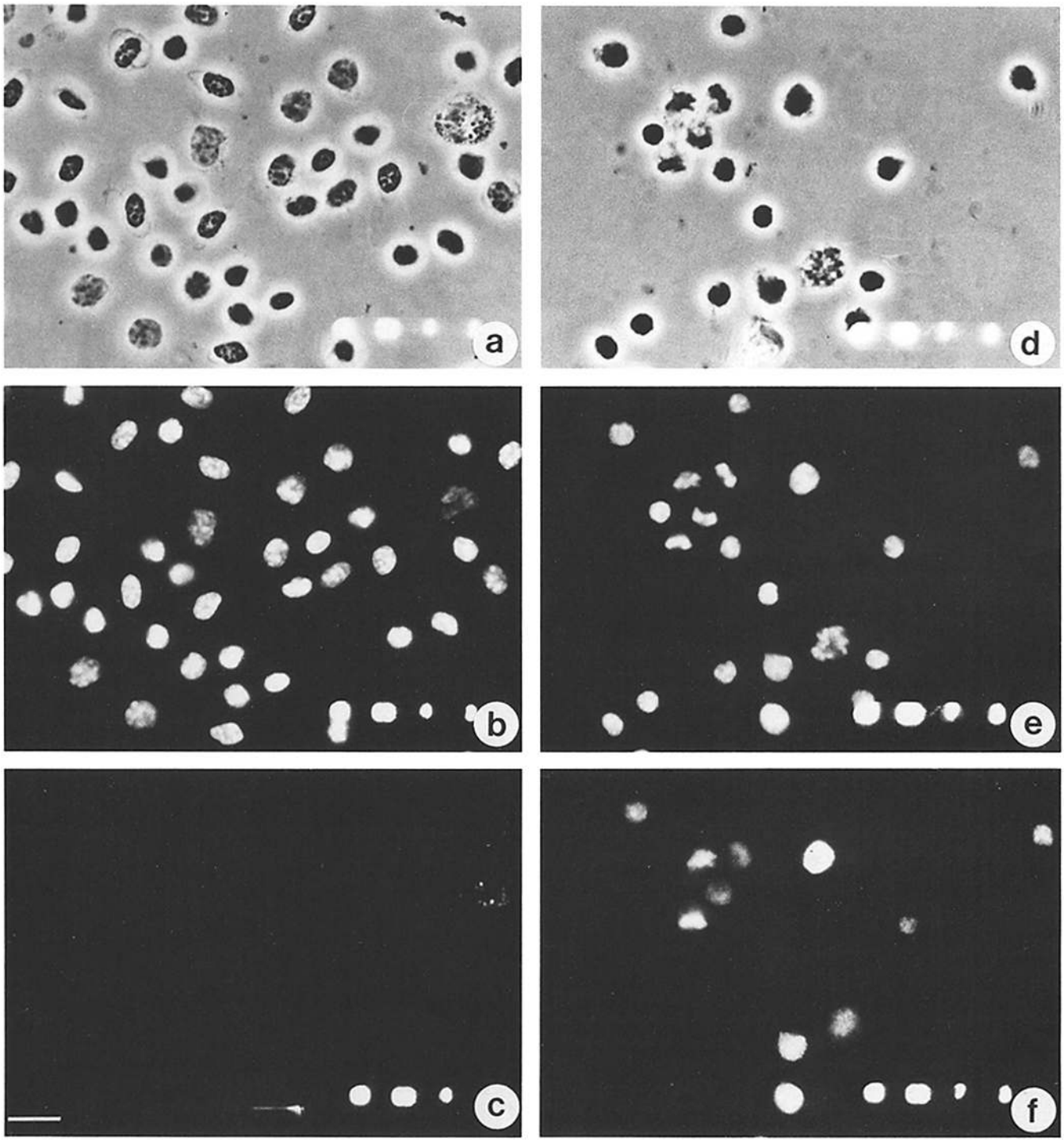

Figure 8. Appearance of topoisomerase II during in vitro activation of chicken lymphocytes. Isolated peripheral blood lymphocytes were cultured in the presence of phytohemagglutinin. At 24 h (*a–c*) or 72 h (*d–f*) after initiation of culture aliquots of cells were centrifuged onto coverslips and processed for indirect immunofluorescence. Phase-contrast (*a*, *d*), DAPI staining of DNA (*b*, *e*), and visualization of topoisomerase II (*c*, *f*). Immunopositive cells appear in the stimulated culture at 26–30 h. Bar, 10 μm.

nome could be constrained in domains as small as 5 kb (assuming that topoisomerase II is found at the base of each loop). This is the inferred size of the loop domains recently determined for the histone gene cluster of *Drosophila* (17).

Earlier reports suggested that topoisomerase II activity correlates with cell proliferation (7, 47, 50), but another study (52) found no such correlation. However, these studies relied solely upon activity assays. As such, the results had to be interpreted with caution since they might not reflect changes in the total amount of enzyme, but could arise from both alterations in the attachment of the enzyme to scaffold/matrix components (affecting the solubilization efficiency), and variations in specific activity occurring across the cell cycle [(15); the latter may result from posttranslational

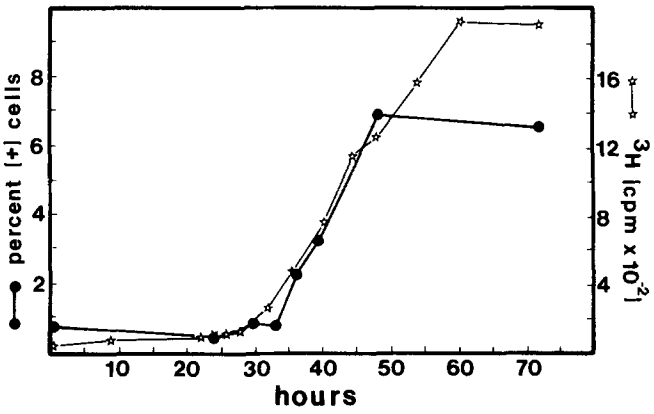

Figure 9. Topoisomerase II antigen appears concurrent with the onset of DNA synthesis. Stimulated lymphocyte cultures (as in Fig. 8) were grown in the presence of 0.5 μCi/ml [^{3}H]thymidine. At the indicated times, aliquots were taken and incorporation of label into acid insoluble material was determined (*stars*). At appropriate time points aliquots were processed for indirect immunofluorescence as in Fig. 8. Random fields were photographed, and the percentage of immunopositive cells determined (*solid circles*). Each point represents the value obtained from analysis of 1,500–3,400 cells.

modifications (1)]. Therefore, we have reexamined the relationship between topoisomerase II content and cell proliferation using a specific antibody probe.

We have now shown that topoisomerase II is present in stoichiometric amounts in nuclei of proliferating cells. When cells cease dividing the enzyme is rapidly lost. The mechanism of removal of topoisomerase II from nonproliferating cells is unknown. Given our results that virtually all topoisomerase II–negative MSB-1 cells had a G_1 DNA content (12), it is tempting to speculate that the enzyme might be degraded at a specific point during G_1, perhaps as part of the progressive chromosome decondensation that precedes DNA replication (40). Our analysis of lymphocyte activation suggests that the enzyme might be synthesized in a cell cycle-regulated manner, with synthesis coupled to DNA replication. In this case, no special mechanism need be invoked to explain the enzyme loss from nonproliferating cells; the failure to reenter a new S-phase would be sufficient. An important test of this model will be a detailed analysis of the synthesis and stability of topoisomerase II across the cell cycle (Heck, M. M. S. and W. C. Earnshaw, manuscript in preparation).

What effect would variation of topoisomerase II levels be expected to have on the organization and activity of the genome? The avian erythroid cells provide an excellent opportunity to address this question, since we have shown that mature erythrocytes contain, if any, <300 copies of the enzyme per cell. Cook and Brazell (5) compared the DNA domain organization of embryonic and adult avian red cells. By measuring the superhelicity of the DNA after gentle dehistonization and centrifugation in sucrose gradients, they concluded that significant alterations of this organization occur during erythropoiesis. Experiments such as these were the basis of the first proposals that the genome of both prokaryotes and eukaryotes is packaged into topologically closed domains in vivo (2, 5). Cook and Brazell (5) observed a steady decrease in the compactness of the genome from 5 d (the earliest sample studied) onward during embryogenesis. In addition, they found that, unlike DNA from all growing cells tested, the DNA of adult erythrocytes behaved as though it was topologically unconstrained. Subsequent workers found that a nuclear matrix fraction cannot be obtained from mature avian erythrocytes (29).

When taken together with our results, these observations suggest that topoisomerase II might be involved in establishment or maintenance of the topological domains. However, considerably more work must be done to test this hypothesis, as the above experiments are open to several alternative explanations. For example, the results of Cook and Brazell (5) could simply arise from the appearance of nucleases in later erythroid cells. In addition, Cook and Brazell (5) did detect some topologically closed domains at times when we have shown the topoisomerase II content of the cells to be virtually nil (at 12 and 18 d, respectively). Finally, it is not clear that topoisomerase II is itself required for stability of the nuclear matrix fraction, since many other nonhistone proteins are also lost during erythropoiesis (41).

The use of developmental systems has enabled us to clarify certain aspects of the relationship between topoisomerase II and genome activity in higher eukaryotes. First, we have shown that high levels of transcription occur in myotubes, in the absence of detectable amounts of the enzyme. This conclusion was derived both from sensitive immunofluorescence experiments and from analysis of single myotubes by simultaneous autoradiography and immunolabeling. The observation is not peculiar to myotubes, since we also found that hepatocyte nuclei lack detectable topoisomerase II antigen (data not shown). We interpret these results to indicate that large amounts of topoisomerase II are not required for maintenance of ongoing transcription. It is possible that the enzyme is required for the establishment of transcriptional activity, a process that presumably occurred in myoblasts before fusion. A similar conclusion was also reached through genetic analysis of yeasts, where mutants lacking topoisomerase II were found to traverse the cell cycle up to mitosis (6, 19, 24, 53). The developmental approach has the advantage, however, that the conclusions derive from observation of unperturbed (wild type) cells.

We have shown that when a naturally arrested population of peripheral blood lymphocytes commences proliferation, topoisomerase II is expressed concomitant with the onset of DNA synthesis. We could find no instances of accumulation of topoisomerase II before the onset of replication, suggesting that the enzyme is unlikely to be a substantial part of any preinitiation complex (unless addition of the enzyme is rate-limiting). We also found few, if any, instances of cells in which replication was observed in the absence of topoisomerase II antigen. It is therefore tempting to assume that topoisomerase II begins to function enzymatically during replication, since synthesis of the enzyme and the DNA appear to be so tightly coupled.

Alternatively, it is possible that the appearance of the enzyme during S phase reflects an "imprinting" of the mitotic chromosome organization during replication. That is, the enzyme might be positioned during replication at the sites where it acts during mitosis. Nelson et al. (36) have recently demonstrated a close association between the enzyme and newly replicated DNA.

The above observations are consistent with the proposal

that a major role of topoisomerase II in higher eukaryotes is to disjoin the sister chromatids at mitosis (49). Preliminary results measuring drug-induced DNA cleavage suggest that the enzyme activity increases significantly at mitosis (15). The large amounts of enzyme present, and its location at the base of radial loops in mitotic chromosomes (9) suggest that decatenation of chromatin loops may in part be regulated by positioning the enzyme at appropriate sites in the chromosome structure.

Regardless of the possible role(s) of topoisomerase II in vivo, we have shown that the level of this protein appears to be a sensitive indication of the proliferative state of a given cell.

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