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Topoisomerase II Expression in Osseous Tissue

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Abstract The molecular mechanisms that mediate the transition from an osteoprogenitor cell to a differentiated osteoblast are unknown. We propose that topoisomerase II (topo II) enzymes, nuclear proteins that mediate DNA topology, contribute to coordinating the loss of osteoprogenitor proliferative capacity with the onset of differentiation. The isoforms topo II-α and -β, are differentially expressed in nonosseous tissues. Topo II-α expression is cell cycle-dependent and upregulated during mitogenesis. Topo II-β is expressed throughout the cell cycle and upregulated when cells have plateaued in growth. To determine whether topo II-α and -β are expressed in normal bone, we analyzed rat lumbar vertebrae using immunohistochemical staining. In the tissue sections, topo II-α was expressed in the marrow cavity of the primary spongiosa. Mature osteoblasts along the trabecular surfaces did not express topo II-α, but were immunopositive for topo II-β, as were cells of the marrow cavity. Confocal laser scanning microscopy was used to determine the nuclear distribution of topo II in rat osteoblasts isolated from the metaphyseal distal femur and the rat osteosarcoma cells, ROS 17/2.8. Topo II-α exhibited a punctate nuclear distribution in the bone cells. Topo II-β was dispersed throughout the interior of the nucleus but concentrated at the nuclear envelope. Serum starvation of the cells attenuated topo II-α expression but did not modulate expression of the β-isoform. These results indicate that the loss of osteogenic proliferation correlates with the downregulation of topo II-α expression. J. Cell. Biochem. 67:451–465, 1997.

Key words: bone; differentiation; nuclear matrix; osteoblast; topoisomerase II

The development of the osteoblast phenotype involves the sequential expression of genes supporting cell division succeeded by genes that mediate the synthesis and maturation of the bone matrix [Stein and Lian, 1993; Pockwinse et al., 1992; Aubin et al., 1995]. Three developmental periods, as distinguished by the expression of specific genes, have been identified in primary cultures of diploid rat calvarial-derived osteoblasts [Stein and Lian, 1993; Stein et al., 1995; Aubin et al., 1995]. These periods progress from (i) proliferation, (ii) extracellular matrix (ECM) synthesis and maturation, to (iii) mineralization.

The molecular events that coordinate the loss of osteogenic proliferative capacity with the successive upregulation of genes associated with ECM synthesis and mineralization are unknown. The occupancy of AP-1 sites in the osteocalcin and alkaline phosphatase gene promoters by fos-jun and/or related proteins have been postulated to suppress the transcription of phenotypic genes in the proliferating osteoblasts [Goldberg et al., 1996; Stein and Lian, 1993; Stein et al., 1995; Owen et al., 1993]. Transcriptional control has been demonstrated to contribute to the regulation of histone H4 during the cell-cycle and during the loss of osteoprogenitor proliferative capacity [Stein and Lian, 1993; Stein et al., 1995; van den Ent et al., 1993;

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Gerbaulet et al., 1992]. Nevertheless, these putative transcriptional mechanisms do not address the question of how multiple genes are coordinately regulated during the temporal differentiation sequence.

Topoisomerase II (topo II) enzymes are attractive candidates for coordinating the loss of osteoprogenitor cell proliferative capacity with the onset of differentiation because (i) they appear to mediate growth and development in other cell types [Chresta et al., 1995; Nakaya et al., 1991; Constantinou et al., 1992; Riou et al., 1993; Sahyoun et al., 1986; Rappa et al., 1990; Ura and Hirose, 1991; Watanabe et al., 1994] and (ii) these proteins regulate the expression of groups of genes through modulating DNA supercoiling and through interactions with trans-acting proteins [Drolet et al., 1994; Wahle et al., 1984; Wang and Lynch, 1993; Kroll et al., 1993; Brou et al., 1993]. The topoisomerases may be part of a general cell mechanism for coordinating global changes in nuclear organization upon the transition from a proliferative to differentiated state [Constantinou et al., 1996]. These enzymes are major structural components of the mitotic chromosome scaffold and interphase nuclear matrix, colocalizing with matrix attachment regions and mediating chromosome loop structure [Giaccone, 1994; Anderson and Roberge, 1992].

The isoforms topo II-α and -β are products of two different genes and their expression at the cell and tissue levels suggest distinctly different roles in differentiation [Giaccone, 1994; Watanabe et al., 1994; Juenke and Holden, 1993; Capranico et al., 1992]. Topo II-α expression is upregulated during mitogenesis and is cell cycle-dependent [Giaccone, 1994; Woessner et al., 1990; Wells et al., 1995]. The expression of this enzyme decreased in cells undergoing differentiation including erythroblasts, myoblasts [Heck and Earnshaw, 1986], and U-937 monoblasts [Drake et al., 1989]. In contrast, topo II-β is expressed throughout the cell cycle and expression often increases when cells plateau in growth [Giaccone, 1994; Woessner et al., 1991].

To determine whether topo II-α and -β are expressed in normal bone tissue, we analyzed rat lumbar vertebrae using immunohistochemical staining. Confocal laser scanning microscopy was employed to examine the nuclear distributions of these proteins in both primary rat osteoblasts and osteosarcoma cells. Our results are consistent with the hypothesis that the downregulation of topo II-α is a significant molecular event in the loss of osteoprogenitor proliferative capacity and the onset of differentiation.

MATERIALS AND METHODS

Reagents

Antibodies used for immunocytochemical, immunohistochemical, and Western analyses included a mouse monoclonal anti-topo II-α, directed against a recombinant human topo II-α peptide (Oncogene Research Products, Cambridge, MA), a mouse monoclonal anti-topo II-α, directed against a recombinant human peptide (University of Utah) [Holden et al., 1994], and polyclonal antiserum to human topo II-β (a generous gift from G. Harker, VA Hospital, Salt Lake City, UT). The two topo II-α antibodies gave identical results in all experiments. Secondary antibodies for immunofluorescence microscopy included anti-mouse immunoglobulin (IgG) conjugated to Texas Red (Jackson ImmunoResearch Labs, West Grove, PA) and anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC; Cappel Res., Durham, NC). Nuclear DNA was stained with 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma, St. Louis, MO). For the staining of tissue sections, we used the Vectastain® ABC kit containing biotinylated, affinity-purified horse anti-mouse and goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). Immunopositive cells were detected using the 3,3′-diaminobenzidine (DAB) substrate kit for peroxidase activity (Vector Laboratories). The peroxidase conjugates to anti-mouse IgG and anti-rabbit IgG (Amersham Life Science, Buckinghamshire, UK) were used as the secondary antibodies for chemiluminescent detection in Western analysis (ECL™, Amersham, Buckinghamshire, England). Reagents for fixation and mounting of cells for immunocytochemistry included acetone (Fisher Scientific, Pittsburg, PA), 20% formaldehyde (Ladd Research Industries, Inc., Burlington, VT), and Aqua Poly-Mount™ (Polysciences, Inc., Warrington, PA). For fixation, decalcification, and embedding of rat osseous tissue we used 10% normal buffered formalin (NBF; Fisher Scientific), Decalcifier 1®, Surgipath paraffin tissue embedding medium (Surgipath Medical Industries, Inc., Grayslake, IL), and Permount™ (Fisher Scientific).

Cell Culture

Primary osteoblasts were derived from the trabecular spongiosa of the distal femur metaphysis of young (70–90 g), male, rats (Charles River Laboratories, Boston, MA) [Alvarez et al., 1997; Onyia et al., 1995, 1997]. This cell preparation expresses the osteoblast phenotype within 4–8 days of culture. Muscle and connective tissue were cut away from femurs of Sprague-Dawley rats; the epiphyseal cap was removed to obtain the subjacent 3 mm section of metaphyseal bone. This section of bone was minced and digested with trypsin for 1 h at 37°C. The released cells were pelleted and resuspended into α-Minimum Essential Medium (MEM) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml amphotericin, 2 mM L-glutamine (GibcoBRL, Grand Island, NY) and 20% fetal bovine serum (FBS; Sigma). The cells were seeded onto sterile glass cover slips in six-well plates (Corning, Cambridge, MA) at an initial density of 2×10^4 cells/9.4 cm^2. Cells were processed for immunocytochemical analysis on day 6 postseeding.

The rat osteosarcoma cells, ROS 17/2.8, a generous gift from Drs. Gideon and Sevgi Rodan (Merck Research Laboratories, West Point, PA) were seeded onto sterile cover slips in six-well plates at an initial density of 7.5×10^3 cells/9.4 cm^2. These cells were grown in Ham's F12 (Gibco BRL) supplemented with 2.36 g/l $NaHCO_3$, 0.118 g/l $CaCl_2 \cdot 2H_2O$, 6.106 g/l HEPES, 100 IU/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml amphotericin, 2 mM L-glutamine and 10% FBS. All cells were maintained in humidified 95% air/5% CO_2 at 37°C.

Immunocytochemistry

Fixation, blocking, and phosphate buffered saline (PBS) washes were all completed at room temperature [Lazarides et al., 1974; Zini et al., 1994]. Cells were fixed for 1 min in 100% acetone and rinsed 3× with 5 min washes of PBS pH 7.4. Cells were then incubated in 1 mg/ml sodium borohydride for 5 min to quench free aldehydes and to decrease autofluorescence [Odgren et al., 1996]. For comparative purposes, additional six-well plates of both cell types were alternatively fixed in 3.7% formaldehyde in PBS for 15 min. Cells were then incubated in 0.1% triton X-100 in PBS for 5 min. The following steps for acetone and formaldehyde fixation were identical. Following a series of PBS washes, cells were blocked in PBS containing 10% FBS and 5% milk for 20 min. Subsequent antibody incubations were carried out in a 37°C humid chamber. Cells were incubated in anti-topo II -α or -β diluted 1:200 and 1:100 respectively, in PBS containing 0.5% bovine serum albumin (P-BSA) for 1 h. Cells were rinsed 3 × 10 min in PBS and incubated in either anti-mouse IgG conjugated to Texas Red diluted 1:2,000, or anti-rabbit IgG conjugated to FITC diluted 1:1,000 for 30 min.

In some experiments, cells were simultaneously incubated in anti-topo II-α and topo II-β. Cells were rinsed in PBS and incubated in anti-mouse IgG conjugated to Texas Red and anti-rabbit IgG conjugated to FITC. Controls for autofluorescence were prepared by omitting both primary and secondary antibodies whereas controls for nonspecific secondary antibody binding were prepared by incubating cells with secondary antibody only. Nuclear DNA was stained by exposing cells to DAPI (5 µg/ml in P-BSA/0.1% Triton X-100) for 5 min.

Immunofluorescence Image Capture and Analysis

Phase contrast and fluorescent digital images were obtained using a Zeiss Axiovert TV light microscope (Thornwood, NY) using either a phase contrast 40× or 100× (NA 1.3) oil-immersion lens. All fluorescent images were acquired using narrow band-pass rhodamine, fluorescein, and DAPI filters and a CCD camera (Photometrics, Inc., Tucson, AZ). Images were captured, processed, and RGB color enhanced using IPLab Spectrum software (Version 3.0, Signal Analytics, Vienna, VA). Mean value intensities from five random fields of topo II-α and -β stained cells and their corresponding controls were obtained per coverslip. Peak primary intensities averaged 2–4× above the mean peak intensities of the secondary controls. Overlay images of cells labelled with both topo II -α and -β antibodies were acquired using the identical exposure conditions for visualizing the individual antigens.

Confocal Laser Scanning Microscopy

High resolution optical sections were obtained using a confocal laser scanning microscope (Odyssey, Noran Instruments, Middleton, WI) equipped with an argon ion laser and a 60× (NA 1.4) oil immersion lens. Cells were illuminated using the 488 nm line for fluorescent excitation and a 515 nm FITC barrier

filter. Eight to ten one micron serial optical sections per nuclei were captured and stored using Image 1 software (Version 4.0.3C, Universal Images, West Chester, PA). The four sequential central planar sections of each series of optical sections were chosen for image presentation. Images were transferred to a Macintosh Quadra 840 AV computer, imported into IPLab Spectrum software for mosaic compilation, and lastly imported into NIH Image software (version 1.59) for application of the color intensity scale. To maximize the true signal to noise ratio, images were corrected for averaged intensities of nonspecific background fluorescence generated from secondary controls.

Immunohistochemistry

Tissue sections. Lumbar vertebrae were isolated from 4–5-week-old male Sprague-Dawley rats (Charles Rivers Laboratories, Boston, MA). Muscle and connective tissue were stripped from the vertebrae and fixed in 10% NBF for 48 h. Following decalcification in Decalcifier 1® solution for 5–7 days, the tissue was thoroughly rinsed in distilled H_2O for 30 min. Specimens were then placed in an automated paraffin processor for infiltration and embedding in paraffin. Tissue sections (5–8 μm) were cut and floated onto positively-charged slides and dried over night at 37°C. Paraffin-embedded tissue sections were melted at 60°C for at least 1 h immediately prior to staining.

Staining. Immunohistochemical staining was performed according to the standard protocol for immunoperoxidase enzyme detection using the Vectastain® ABC technique (Vector Laboratories, Burlingame, CA) as modified by Holden et al. [1994]. The staining procedure was performed at room temperature. Tissue sections were deparaffinized in two successive 10 min washes of xylene, followed by two washes in 100% ethanol. Following a brief wash in PBS containing 0.1% Tween-20 (PBS-T), pH 7.4, slides were placed in freshly prepared 1.5% H_2O_2 in methanol for 20 min to inhibit endogenous peroxidase activity. In a separate staining dish, 500 ml of 10 mM sodium citrate buffer, pH 6.0, was heated by microwave at 100% power until just boiling. Slides were immediately immersed in the hot sodium citrate buffer for 30 min, and then rinsed in PBS-T for 10 min. All subsequent incubation steps were completed in a humid chamber. Tissue sections were blocked in 10% normal goat serum in PBS for 20 min. Primary antibodies to topo II-α or -β were then applied to the sections for 1 h at a dilution of 1:100 and 1:200 in PBS, respectively. Controls for nonspecific secondary binding were incubated without primary antibody. Secondary biotinylated antibodies were applied at concentrations of 2 μg/ml PBS for the anti-mouse IgG or 3 μg/ml PBS for the anti-rabbit IgG for 30 min. Prior to color development, slides were incubated in standard avidin-biotinylated horseradish peroxidase solution (Vector Laboratories) for 1 h. Peroxidase activity was detected by a 2–4 min incubation in DAB substrate solution. Slides were counterstained for 1 min in hematoxylin, processed through a series of ethanol dehydrations, and permanently mounted in Permount™.

Protein extraction. Chromatin fractions of ROS 17/2.8 cells were obtained from a standard sequential extraction protocol as previously described [Bidwell et al., 1996].

Protein analysis. Chromatin extracts from ROS 17/2.8 were analyzed by Western blotting. Total protein concentration of chromatin extracts was measured using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Protein extracts (50 μg/lane) were electrophoresed on 5% 1-D SDS-polyacrylamide gels and then the proteins transferred onto a PVDF membrane (Millipore Corp., Bedford, MA) by the semidry method [Ausubel et al., 1987]. Membranes were blocked overnight at room temperature in 5% powdered milk in TBS-T. For topo II-α analysis, blots were incubated with 1:1,000 primary and secondary antibodies. For topo II-β measurements, the membranes were incubated with 1:500 primary antibody and 1: 15,000 secondary antibody. Immunoblots were developed using chemiluminescence (ECL™, Amersham).

Serum starvation. At day 5 postseeding, primary spongiosa cells were exposed to α-MEM medium containing 0% FBS. Controls were replenished with α-MEM medium supplemented with 10% FBS. After 24 h, cells from both treatments were fixed and processed for immunocytochemical analysis of topo II-α and -β expression and stained with DAPI. For quantitative analysis, five fields from nine control and nine serum starved slides were captured using identical exposure conditions. Total cell number per field was determined by counting of DAPI stained nuclei. Topo II-α and -β immunopositive cells were defined as those that had

a fluorescent intensity greater than the mean peak intensity plus one s.d. of the secondary controls. The percentage of immunopositive cells was determined by normalization to the total cell number per coverslip. Differences between control and serum starved cells were analyzed using the student's *t*-test.

DNA synthesis. Cell proliferation state was determined by measurement of [^{3}H]-thymidine uptake as previously described [Onyia et al., 1997]. Briefly, primary spongiosa cells were seeded in 96 well plates (1.2×10^4 cells/well) in α-MEM containing 10% FBS. After 24 h recovery, cells were exposed to serum-free (0% FBS) α-MEM medium, or medium containing 10% FBS, in the presence of 10 μCi/ml [^{3}H]-thymidine (82.7 Ci/mmol; Amersham International, Arlington Heights, IL) for an additional 24 h. After 24 h, the medium was removed, the cells were harvested, lysed, and [^{3}H]-thymidine incorporation was assayed by liquid scintillation [Onyia et al., 1997]. The results were obtained in DPM and calculated as the mean ± SEM of five experiments.

RESULTS

TopoII -α and -β Protein Expression in Normal Rat Bone

Immunohistochemical analysis of decalcified, paraffin embedded tissue sections of rat lumbar vertebrae indicated that topo II-α and -β were expressed in normal bone (Fig. 1A–D). Topo II-α was selectively expressed in the bone marrow cell population, particularly near the growth plate (Fig. 1A,C). This protein was occasionally observed in the proliferative zone of the cartilage growth plate (Fig. 1E). Topo II-α expression was strikingly absent in mature osteoblasts and osteocytes. None of the large ovoid cells lining the trabeculae were immunopositive, nor were the nuclei of the osteocytes embedded in these bone spicules (Fig. 1A,C). Although nearly all topo II-α immunopositive cells showed precise nuclear staining, cytoplasmic staining was detected in a few bone marrow cells, consistent with previous results in non-osseous proliferating cells [Petrov et al., 1993; Swedlow et al., 1993].

Topo II-β was expressed in the bone marrow cells as well as the mature osteoblasts and most osteocytes (Fig. 1B,D). In contrast to topo II-α, the large ovoid cells lining the trabeculae exhibited an intense nuclear staining and about half of the osteocytes within the bone spicules were immunopositive (Fig. 1B,D). Topo II-β was also expressed by the cells of the proliferative zone of the cartilage template (Fig. 1F).

Results from Western analyses of the chromatin fractions of ROS 17/2.8 cells were consistent with previous histochemical studies using these antibodies [Bauman et al., 1997; Lynch et al., 1997]. Both of our topo II-α antibodies detected a major band at 170 kD on immunoblots of ROS 17/2.8 extracts and a yeast extract containing recombinant human topo II-α (Fig. 2). As in earlier studies [Bidwell et al., 1996; Bauman et al., 1997], anti-topo II-β detected antigens of 180 kD and 100 kD (Fig. 2), the 100 kD band is thought to be a breakdown product of the enzyme [Bauman et al., 1997].

Topo II-α and -β Exhibit Distinct Nuclear Distributions

Immunofluorescence microscopy of cultured primary spongiosa cells and ROS 17/2.8 osteosarcoma cells exclusively labelled for either topo II-α or -β indicated that topo II-α exhibited an intranuclear punctate distribution and was not observed in all cells (Fig. 3B,E). In contrast, topo II-β was dispersed throughout the nucleus but concentrated at the nuclear envelope and was expressed in all cells in both primary osteoblast and osteosarcoma cultures (Fig. 3C,F). Consistent with our previous studies using Western analysis [Bidwell et al., 1996], topo II-α was detected in a higher percentage of osteosarcoma cells as compared to the primary spongiosa cultures (data not shown).

Simultaneous detection of topo II-α and -β expression in osteogenic cells double-labelled with topo II-α and -β antibodies demonstrated that with the coexpression of both isoforms, some spatial overlap of these two enzymes occurred (Fig. 4C,F). Neither topo II-α or -β appeared to be excluded from the nucleolus, although in some of the ROS 17/2.8 cells peak levels of topo II-α were observed in these regions (Figs. 3 and 4).

To confirm the intranuclear distributions of topo II-α and -β first observed by light microscopy, serial optical sections (1 μm) of primary spongiosa and ROS 17/2.8 osteosarcoma cells were obtained using high resolution confocal laser scanning microscopy. Images of serial optical sections verified that topo II-α protein exhibited a punctate localization and was distributed throughout the volume of the nucleus (Fig. 5A–D). This enzyme was particularly con-

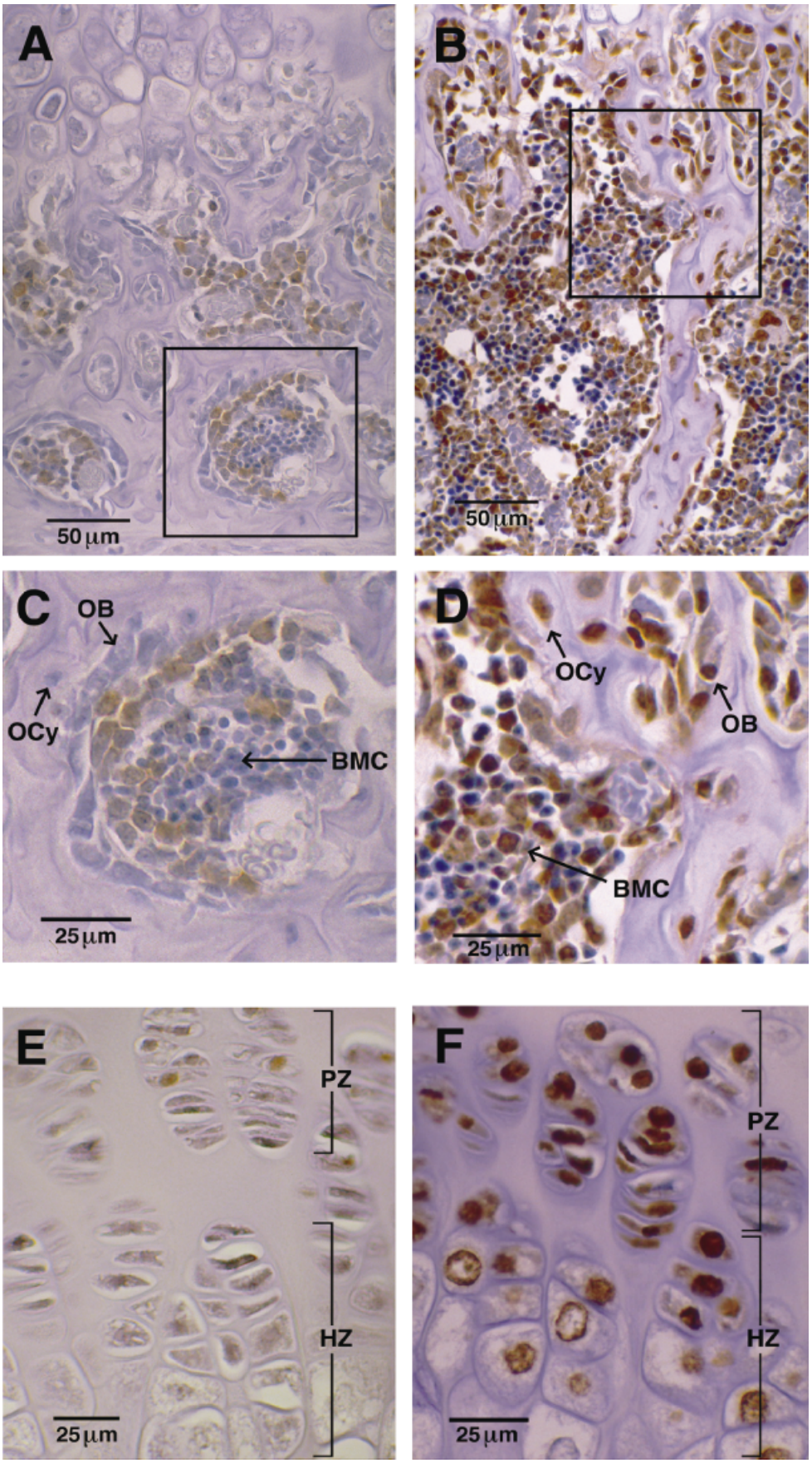
A
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Figure 1.

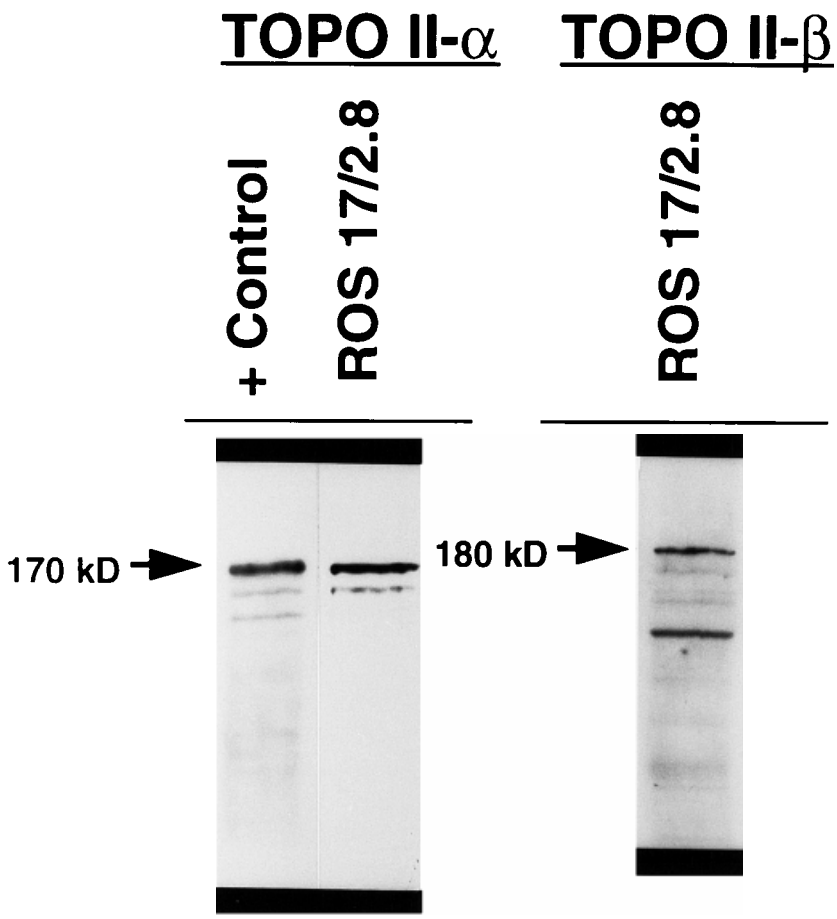

Fig. 2. Western analysis of topo II-α and -β. See Materials and Methods for details of analysis. **A:** Topo II-α: The positive control was recombinant human protein expressed in yeast. The major 170 kD band is topo II-α. Minor bands were detected at 160 kD and 140 kD in both the positive control and cell extract; these may be proteolytic fragments. **B:** Topo II-β: The 180 kD band is topo II-β. The 100 kD is thought to be a breakdown product [Bauman et al., 1997].

centrated in the central planar sections (Fig. 5B,C). Optical sections of osteoblast and osteosarcoma nuclei stained for topo II-β indicated a similar concentration of protein near the nuclear planar center, a comparatively more uniform dispersion of protein throughout these focal planes, and peak concentrations at the nuclear envelope (Fig. 6A–D).

Fig. 1. Immunohistochemical detection of topo II-α and -β in decalcified, paraffin-embedded tissue sections of rat lumbar vertebrae (male, Sprague-Dawley, 4–5 weeks of age). Immunopositive cells were detected with immunoperoxidase enzyme detection system using Vectastain® ABC technique. Peroxidase activity was detected by DAB (brown). **A,C:** Topo II-α expression in lumbar vertebra. Some cells of the bone marrow cavity (BMC) stain for enzyme expression. Mature osteoblasts (OB) and osteocytes (OCy) show no expression of topo II-α. **E:** Topo II-α was observed in the proliferative zone (PZ) of the cartilage. **B,D:** Topo II-β expression in lumbar vertebra. This isoform is expressed by cells of the marrow cavity and the mature osteoblasts and osteocytes. **F:** Topo II-β was also observed in the PZ of the cartilage. Some staining was evident in the dead or dying cells of the HZ.

Serum Starvation of Primary Spongiosa Osteoblasts Attenuates DNA Synthesis and Topo II-α Protein Expression But Does Not Influence Topo II-β Expression

To determine whether there is a differential coupling between the expression of topo II-α and -β and the proliferative state of the osteoblast, we compared the level of expression of these two proteins in serum starved primary spongiosa cells. Bone cells starved of serum for 24 h exhibited an ~90% decrease in both thymidine uptake and topo II-α expression as compared to controls (Table I). We observed that ~95% of the cells in both the control and serum starved cultures expressed topo II-β.

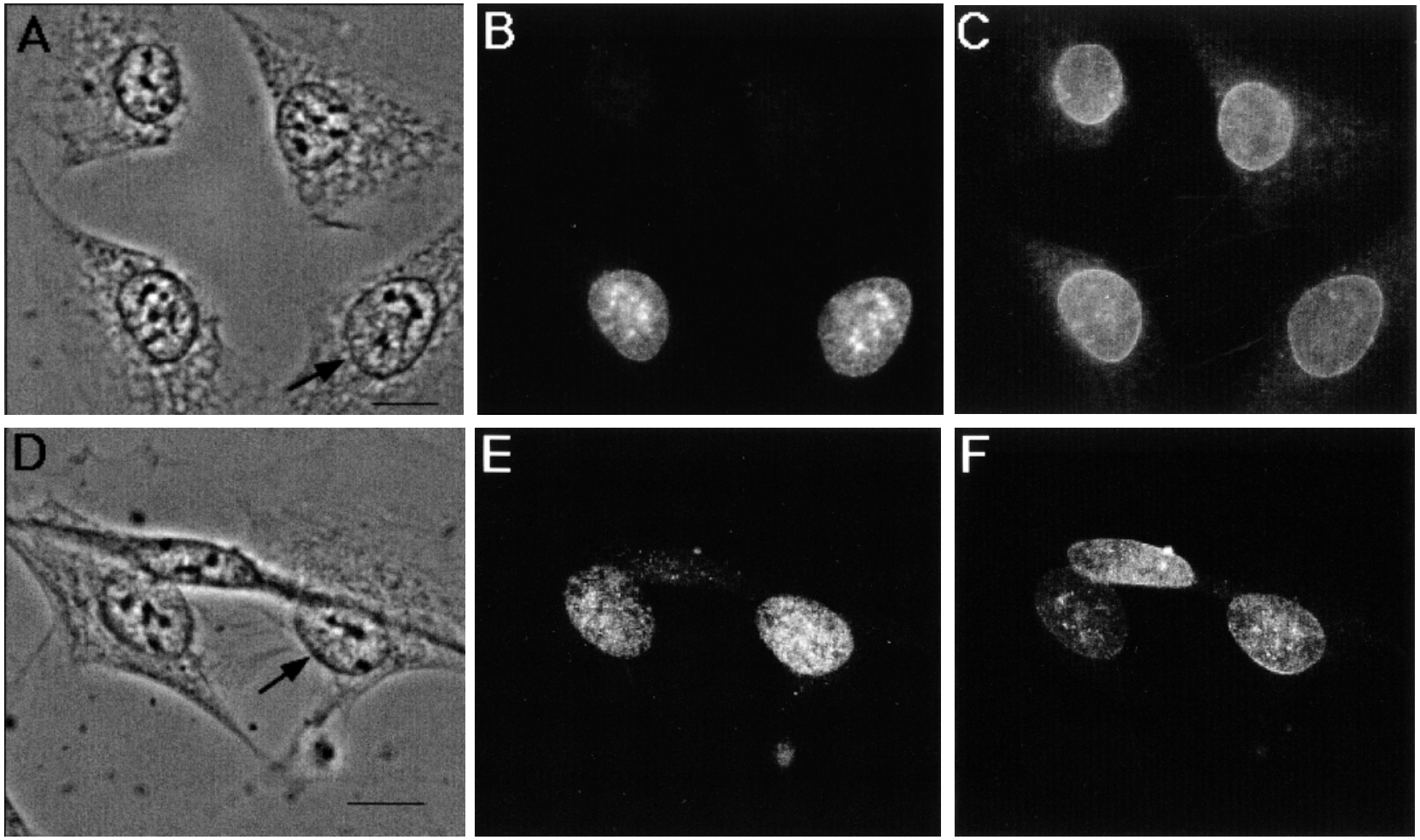

Fig. 3. Localization of topo II-α and -β in cultured osteogenic cells. A–C: Phase contrast **(A)** image of ROS 17/2.8 cells immunolabelled for topo II-α **(B)** and -β **(C).** Note the more punctate nuclear distribution of the α-isoform as compared to the β-enzyme. In some of the ROS 17/2.8 cells, topo II-α appeared to aggregate in the nucleolus. Topo II-β was concentrated at the nuclear envelope. Also, topo II-β was uniformly expressed in all cells in contrast to topo II-α. D–F: Phase contrast **(D)** image of primary spongiosa cells immunolabelled for topo II-α **(E)** and -β **(F).** Arrows (A,D) indicate select nuclei enlarged and color enhanced in Figure 4. Cells fixed in 3.7% formaldehyde showed nearly identical patterns of topo II-α and -β localization. Scale bar = 10 μm.

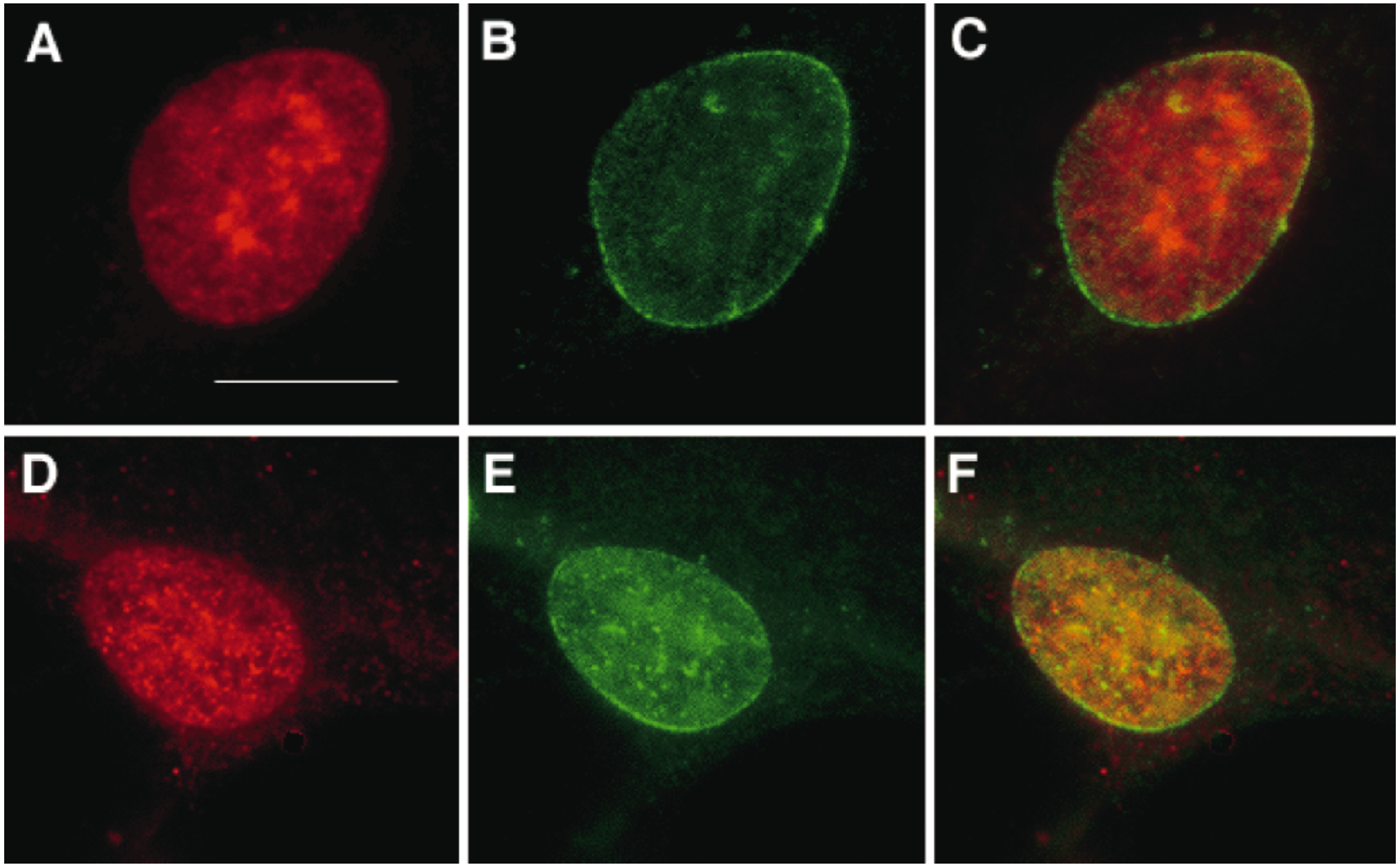

Fig. 4. Color overlay images of topo II immunolabelled ROS 17/2.8 cells (A–C) and primary spongiosa cells (D–F). **A,D:** Topo II-α expression (red). **B,E:** Topo II-β expression (green). **C,F:** Color overlay demonstrating spatial relationship between the topo II isoforms (red = α-isoform; green = β-isoform; yellow = both isoforms). Phase contrast of both nuclei are presented in Figure 3 (bold arrows). Scale bar = 10 μm.

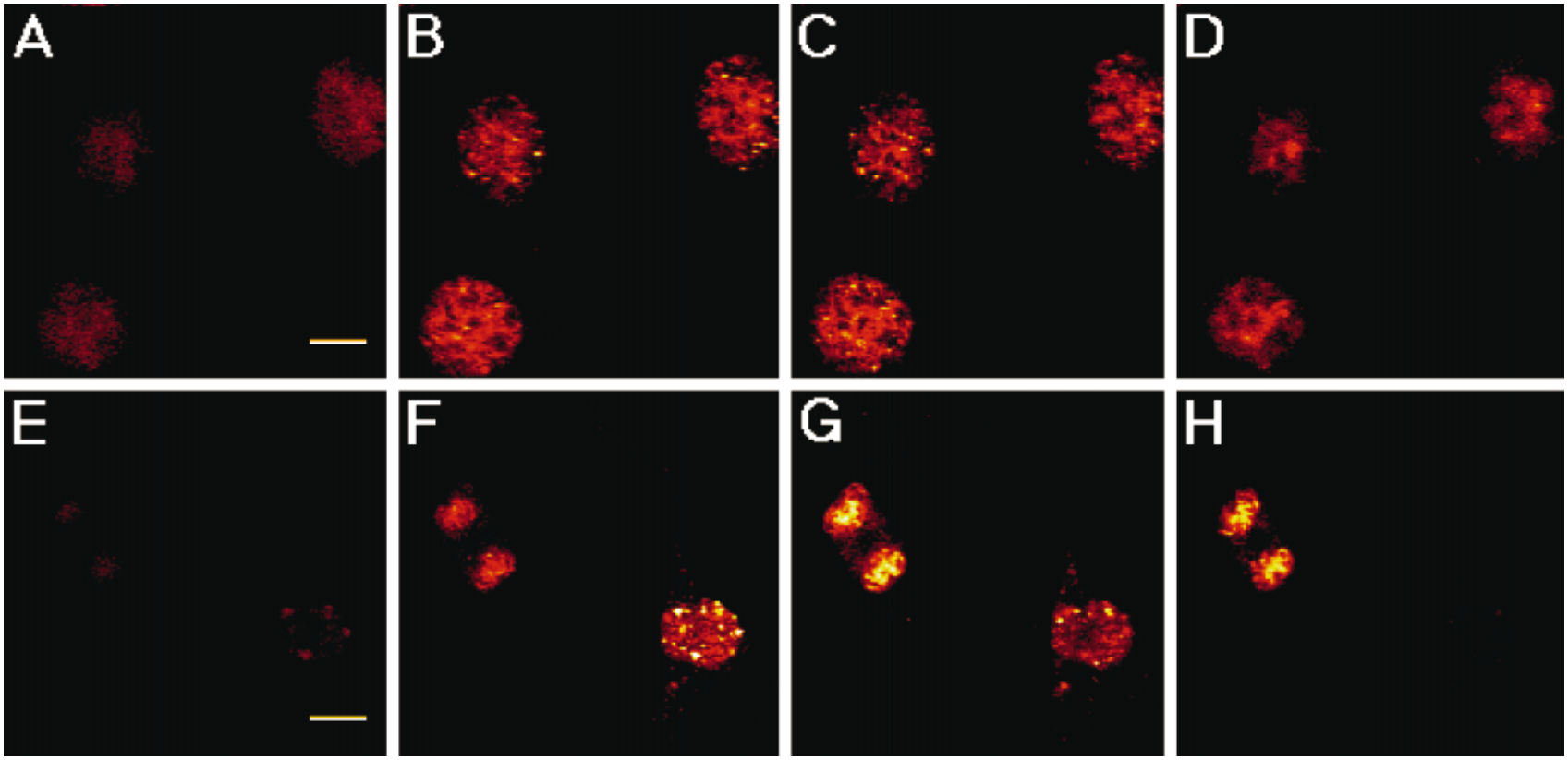

Fig. 5. One micron serial optical sections of ROS 17/2.8 nuclei **(A–D)** and primary spongiosa nuclei **(E–H)** showing the distribution of topo II-α. The fields of the primary osteoblasts (E–H) include two cells undergoing mitosis and an interphase cell. Topo II-α appears punctate throughout the nucleus during interphase, and appears to localize with the chromosome scaffold during mitosis. Maximal fluorescent intensity is indicated by yellow and white. Images shown represent the four sequential central planar sections from each series of optical sections. Scale bar = 10 μm.

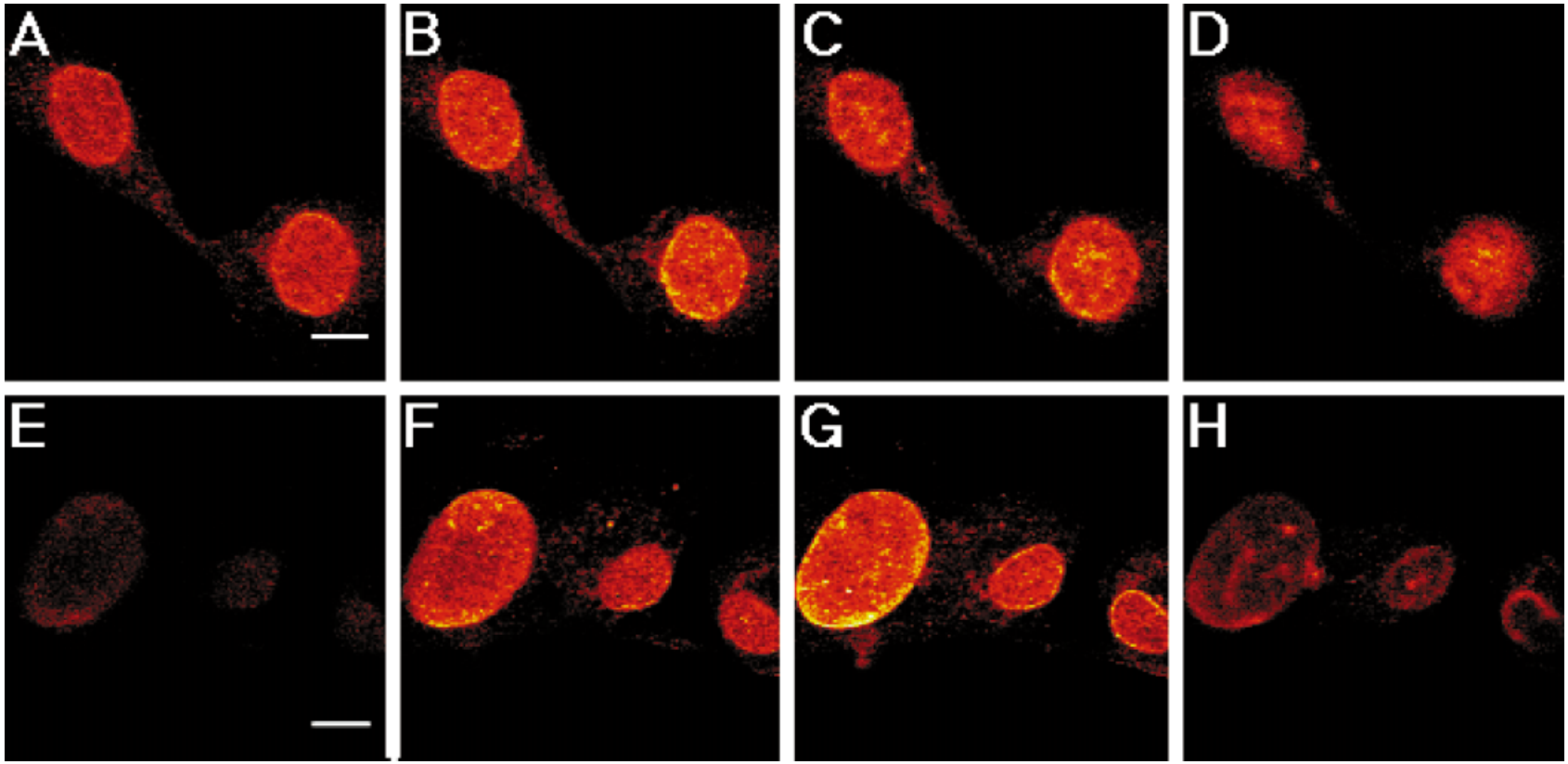

Fig. 6. One micron serial optical sections of ROS 17/2.8 nuclei **(A–D)** and primary spongiosa nuclei **(E–H)** showing the distribution of topo II-β. Topo II-β is uniformly dispersed throughout the interior of the nucleus, with peak intensities at the nuclear envelope. Maximal fluorescent intensity is indicated by yellow and white. Images shown represent the four sequential central planar sections from each series of optical sections. Scale bar = 10 μm.

TABLE I. Serum Starvation Data

	Cell count	Topo II-α positive cells [%]	Topo II-β positive cells [%]	DNA synthesis (DPM)
Control	121.8 ± 4.7	16 ± 1.4	94.4 ± 1.0	144,380 ± 9,895
No serum	83.5 ± 5.4	2.1 ± 1.3	98.2 ± 0.7	15,054 ± 1,233
	($P = 0.0001$)	($P = 0.0001$)	($P = 0.006$)	($P = 0.0001$)

DISCUSSION

The present data is significant because it demonstrates the differential expression of topo II-α and -β in normal bone. Although the literature is replete with studies on the expression of these proteins in tumors, there is comparatively little information on the presence of the topoisomerases in normal tissue. We have also determined that these enzymes exhibit distinct nuclear distributions in osteoblasts. These observations suggest that topo II-α and -β have distinct, but perhaps related, functions in the development of the osteoblast phenotype.

In osseous tissue, topo II-α expression appears coincident with proliferation of osteoprogenitor cells. Topo II-α was detected in the bone marrow space of vertebral tissue sections, which includes hemopoietic, bone marrow, and osteoprogenitor cells. This protein was also expressed in the cartilage proliferative zone of these samples. Topo II-α was not observed in terminally differentiated bone cells including the osteoblasts along the trabeculae and the osteocytes. Topo II-β, however, was detected in cells of the marrow cavity, the cartilage proliferative zone, and the mature osteoblasts and osteocytes. Additionally, serum starvation of primary spongiosa cultures decreased the number of osteoblasts expressing topo II-α, and thymidine uptake by ~90% but had little affect on topo II-β expression. In an earlier study we observed that topo II-α expression was upregulated in the rat osteosarcoma cell line ROS 17/2.8, as compared to the primary cultures of spongiosa osteoblasts; topo II-β expression, however, was equivalent between the cancer cells and primary osteoblasts [Bidwell et al., 1996].

To date, the few studies on topo II expression in normal tissues indicate that the α isoform is restricted to proliferative tissues such as testis (spermatocytes), the germinal centers in lymph nodes, tonsils, spleen, and proliferative endometrium [Bauman et al., 1997; Zandvliet et al., 1996; Watanabe et al., 1994; Juenke and Holden, 1993; Capranico et al., 1992]. This enzyme is absent in terminally differentiated tissues such as cerebral cortex, skeletal muscle, and nerve [Bauman et al., 1997]. Topo II-β, however, is ubiquitously expressed in tissues with high cell turnover, e.g., endometrium and bowel mucosa, and in differentiated tissues such as cerebellum, myometrium, and pancreas [Bauman et al., 1997; Zandvliet et al., 1996; Juenke and Holden, 1993].

Further support for the involvement of topo II-α in mitogenesis includes its cell cycle-dependent expression and phosphorylation [Rattner et al., 1996; Wells et al., 1995; Woessner et al., 1991]. Topo II-α expression was limited to the late S and G_2-M phases of synchronized NIH-3T3 cells but topo II-β levels were constant once the cells entered the cell cycle [Woessner et al., 1991]. When exponentially growing NIH-3T3 cells were induced to enter G_0 by serum starvation, topo II-α expression decreased in parallel with the loss of cells from the S and G_2-M phases but topo II-β expression was not affected [Woessner et al., 1991]. Topo II-α expression is often upregulated in tumor cells, may serve as a proliferation marker in malignant tissues [Bauman et al., 1997; D'Andrea et al., 1994; Holden et al., 1992, 1994, 1995], and is a target for chemotherapy [Beck et al., 1994].

The ubiquitous distribution of topo II-β in both proliferative and mature tissues, its constant expression throughout the cell cycle, and its comparatively tighter association with the nuclear matrix than the α-isoform [Woessner et al., 1991; Zini et al., 1994], suggests an essential role in mediating the integrity of nuclear structure and chromosome loop organization [Drake et al., 1989]. Whereas topo II-α and -β form heterodimers in cells [Biersack et al., 1996], the expression of both isoforms may mediate a nuclear topology distinct from that organized by topo II-β alone.

The distinct distributions of topo II-α and -β in the osteoblast nucleus are consistent with data on other cell types [Zini et al., 1994; Petrov et al., 1993] and further support different functional roles for these two isoforms. The punc-

tate distribution of topo II-α throughout the osteoblast nucleoplasm conforms with the pattern of expression observed in Chinese hamster fibroblasts [Petrov et al., 1993], HeLa cells, and K562 human myeloid cells [Zini et al., 1994]. The distribution of the β-isoform appears more cell-specific. Topo II-β was localized to the nucleolus in HeLa cells, K562 cells, and in some tumor tissues [D'Andrea et al., 1994; Zini et al., 1994; Negri et al., 1992]. Our own data indicated that topo II-β was dispersed throughout the nucleoplasm but concentrated near the nuclear envelope of the osteoblasts and osteosarcoma cells. We did observe the β-isoform in the nucleolus of those optical sections containing this organelle. This is similar to the nuclear distribution of this enzyme in Chinese hamster fibroblasts [Petrov et al., 1993]. In these cells, topo II-β (and topo II-α) were present in the nucleoplasm and the nucleolus. In the nucleoplasm both enzymes frequently localized at the periphery of heterochromatin regions [Petrov et al., 1993]. Therefore, although topo II-β appears to be ubiquitously expressed by both proliferating and terminally differentiated tissues, its nuclear distribution may vary between cell type. Conversely, topo II-α expression is largely restricted to proliferating tissues, but exhibits a similar nuclear distribution in most cells.

Topo II-α may be part of a general cell mechanism that mediates proliferation independently of differentiation, or may act as a permissive mediator that coordinates the transition between a dividing and non-dividing, maturing cell. The capacity of topo II-α for modulating gene expression both locally and globally through interactions with DNA and transacting proteins [Drolet et al., 1994; Wahle et al., 1984; Wang and Lynch, 1993; Wang and Dröge, 1996; Kroll et al., 1993; Brou et al., 1993] places this enzyme in a unique position to orchestrate the myriad of changes in gene expression necessary for switching from a genetic program supporting proliferation to one mediating differentiation. Indeed, recent data indicate that altering topoisomerase activity has a potent effect on the initiation of differentiation in numerous cells [Constantinou et al., 1996; Rashid and Basson, 1996; Chresta et al., 1995; Nakaya et al., 1991; Constantinou et al., 1992; Riou et al., 1993; Sahyoun et al., 1986; Rappa et al., 1990; Ura and Hirose, 1991].

When considering the role of the topoisomerases in osteoblast development, the question as to how ubiquitously expressed proteins can contribute to the differentiation pathway of a specific tissue must be addressed. The immediate early gene c-fos is expressed in all tissues, yet this protein appears to exert specific effects on the expression of the osteoblast phenotype [Grigoriadis et al., 1993]. Homozygous transgenic mice overexpressing c-fos from an H-2Kb class I MHC promoter developed osteosarcomas with a short latency period [Grigoriadis et al., 1993]. Clonal osteoblast-like cells derived from these animals exhibited an abrogation of the 1,25-dihyroxyvitamin D_3-regulated expression of osteocalcin and alkaline phosphatase. Recent data support a role for AP-1 protein as a regulator of bone cell development by suppressing the activity of osteoblast-specific promoters during the proliferative period [Goldberg et al., 1996; Yamauchi et al., 1996; McCabe et al., 1995]. Topo II-α could conceivably play a role in this phenotype suppression model since this enzyme contains a leucine zipper protein dimerization motif that mediates interactions with c-jun [Kroll et al., 1993]. The molecular mechanisms that mediate the transition between an osteoprogenitor cell and a non-dividing, maturing osteoblast, appear to exploit both tissue-specific, as well as general cell machinery. The topoisomerases likely participate in the normal development of bone.

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