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Parathyroid Hormone Regulates the Expression of Rat Osteoblast and Osteosarcoma Nuclear Matrix Proteins

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Abstract Parathyroid hormone (PTH) alters osteoblast morphology. How these changes in cell shape modify nuclear structure and ultimately gene expression is not known. Chronic exposure to rat PTH (1-34) [10 nM] attenuated the expression of 200, 190, and 160 kD proteins in the nuclear matrix-intermediate filament subfraction of the rat osteosarcoma cells, ROS 17/2.8 [Bidwell et al. (1994b): Endocrinology 134:1738–1744]. Here, we determined that these same PTH-responsive proteins were expressed in rat metaphyseal osteoblasts. We identified the 200 kD protein as a non-muscle myosin. Although the molecular weights, subcellular distribution, and half-lives of the 190 and 160 kD proteins were similar to topoisomerase II-α and -β, nuclear matrix enzymes that mediate DNA topology, the 190 and 160 kD proteins did not interact with topoisomerase antibodies. Nevertheless, the expression of topoisomerase II-α, and NuMA, a component of the nuclear core filaments, was also regulated by PTH in the osteosarcoma cells. The 190 kD protein was selectively expressed in bone cells as it was not observed in OK opossum kidney cells, H4 hepatoma cells, or NIH3T3 cells. PTH attenuated mRNA expression of the PTH receptor in our cell preparations. These results demonstrate that PTH selectively alters the expression of osteoblast membrane, cytoskeletal, and nucleoskeletal proteins. Topoisomerase II-α, NuMA, and the 190 and 160 kD proteins may direct the nuclear PTH signalling pathways to the target genes and play a structural role in osteoblast gene expression.

Key words: tissue matrix, primary spongiosa, PTH-induced downregulation, topoisomerase, NuMA

Acute and chronic exposures of cultured bone cells to parathyroid hormone (PTH) selectively alter the organization and structure of the osteoblast membrane and cytoskeleton [Partridge et al., 1994; Sato and Rodan, 1989]. PTH receptor coupling to membrane-associated G-proteins and effector molecules is altered upon hormone binding and continuous exposure decreases receptor number [Nissenson et al., 1993]. Transient changes in rat osteoblast shape, induced by a five minute exposure to PTH, was associated with depolymerization of actomyosin and a redistribution of myosin to the perinuclear region [Egan et al., 1991a]. In contrast, chronic exposure to PTH attenuated de novo synthesis of vimentin and alpha-actinin and decreased the polymerized fractions of actin, alpha-tubulins and alpha-actinin in human osteoblasts [Lormi and Marie, 1990].

How the PTH-induced signal cascade of soluble messengers and cytoskeletal reorganization alter nuclear structure and change osteoblast gene expression is not known. The DNA within the eukaryotic nucleus is organized into topological-constrained loop domains providing the structural basis for tissue- and phenotypic-specific gene expression [Pienta et al., 1991; Getzenberg et al., 1990]. This organization is mediated by the nuclear matrix [Pienta et al., 1991; Getzenberg et al., 1990] which is operationally defined as the proteinaceous substructure that resists nuclease digestion and high-salt extraction [Berezney and Coffey, 1975] and includes the lamin-nucleopore complex, a dense fibrillogranular lattice of ribonucleoproteins and hnRNA filaments, and the chromosome scaffold [Razin and Gromova, 1995; Nickerson and Penman, 1992; Fey et al., 1984; Mirkovitch et al., 1984].

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The nuclear matrix is a likely candidate for ultimately transducing extracellular signals initiated at the cell membrane into alterations of nuclear organization and gene expression. This nuclear scaffold is linked to the cell periphery by the tissue matrix, the substructure of the cell that includes components of the extracellular matrix, cell-substrate, and cell-cell adhesion receptors, microfilaments, microtubules, intermediate filaments, lamins, and the nuclear matrix [Getzenberg et al., 1990]. The nuclear matrix plays a significant role in DNA replication [Berezney, 1991], the processing and transport of RNA transcripts [Carter et al., 1993], and transcriptional regulation [Nakagomi et al., 1994]. Its protein composition and organization reflects and mediates changes in cellular growth, differentiation, and transformation [Pienta et al., 1991; Getzenberg et al., 1990; Bidwell et al., 1994a; Dworetzky et al., 1990]. Rat osteoblast nuclear matrix protein composition was specific to stages of differentiation including proliferation, extracellular matrix maturation, and extracellular mineralization [Dworetzky et al., 1990]. NMP2 is a 38 kD, variant AML/PEBP2/*runt domain* nuclear matrix protein, expressed in rat osteoblasts, that associates with sequence-specificity to the regulatory region of the osteocalcin promoter [Merriman et al., 1995].

Chronic exposure to rat PTH (1-34) attenuated the expression of 200, 190, and 160 kD proteins in the nuclear matrix-intermediate filament and chromatin subfractions of ROS 17/2.8 cells [Bidwell et al., 1994b]. In the present study we determined that these same proteins were also expressed in primary spongiosa cells of the metaphyseal distal femur of young rats. Biochemical and Western analyses indicated that the 200 kD protein was a non-muscle myosin. Although the molecular weights, subcellular distribution, and half-lives of the 190 and 160 kD proteins were similar to topoisomerase II-α and -β, nuclear matrix enzymes that mediate DNA topology, the 190 and 160 kD proteins did not interact with topoisomerase II antibodies. Nevertheless, PTH did regulate the expression of topoiosmerase II-α in the osteosarcoma cells. Finally, exposure to PTH attenuated mRNA expression of the PTH receptor in our cell preparations. These results demonstrate that PTH coordinately reorganizes the osteoblast tissue matrix including specific nuclear matrix proteins that may mediate hormone-induced alterations in nuclear structure and changes in gene expression.

MATERIALS AND METHODS

Reagents

For immunoblotting we used antibodies to β-actin (Sigma Chemical Company, St. Louis, MO), NuMA and topoisomerase II-α (Oncogene Science, Inc., Uniondale, NY), and non-muscle myosin (Biomedical Technologies Inc., Stoughton, MA). The antibody to topoisomerase II-β (a generous gift from G. Harker, VA Hospital, Salt Lake City, UT) was a rabbit polyclonal to the human enzyme. The peroxidase conjugates to anti-rabbit IgG and anti-mouse IgG (Amersham Life Science, Buckinghamshire, UK) were used as the secondary antibodies for chemiluminescence detection (ECL™, Amersham).

The protease inhibitors aprotinin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride (PMSF) (Boehringer Mannheim, Indianapolis, IN), and benzamidine · HCl (Aldrich Chemical Co., Milwaukee, WI) were used in the preparation of whole cell lysates. The hormone rat PTH(1-34) [rPTH(1-34)] was obtained from Bachem (King of Prussia, PA). Reagents for the extraction buffers were obtained from Fisher Chemical Co. (Pittsburgh, PA) and Sigma.

Cell Culture

Primary osteoblasts were derived from the trabecular spongiosa of the distal femur metaphysis of young rats (Charles River Laboratories, Boston, MA) [Long et al., 1995]. Briefly, muscle and connective tissue were stripped from femurs of male, Sprague-Dawley rats (approximately 70–90 g); the epiphysis was removed and the subjacent 3 mm section of metaphyseal bone resected. 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 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). Cells derived from 4 femurs were seeded into a single T-150 flask or the cells from 1 femur into a T-25 flask (Corning, Corning, NY).

The rat osteosarcoma cells, ROS 17/2.8, a generous gift from Drs. Gideon and Sevgi Rodan (Merck Research Laboratories, West Point, PA),

and the OK opossum kidney cells, a gift from Dr. James McAteer (Department of Anatomy, Indiana University, Indianapolis) 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. The rat H4-II-E-C3, hepatoma cells were from the American Type Culture Collection (Rockville, MD) and grown in D-MEM (GibcoBRL) supplemented with penicillin, streptomycin, amphotericin, glutamine, and FBS as described above. The NIH3T3 mouse fibroblasts were obtained from Eli Lilly Co (Indianapolis, IN) and grown in MEM medium (GibcoBRL) supplemented as described for D-MEM medium. All cells were maintained in humidified 95% air/5% CO_2 at 37°C.

At near confluence, cells were treated with rat PTH(1-34) [10 nM] or the same volume of vehicle (10 mM acetic acid) for the times specified (see Results).

Cell Labelling

One hour or 24 h before harvest, cells were transferred to MEM without methionine (GibcoBRL), supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 25μg/ml amphotericin, 2 mM L-glutamine, 10% FBS (dialyzed against PBS), and 25 μCi/ml of ^{35}S-methionine (Amersham).

Pulse-Chase Experiments

Cells were labelled for 1 h as described above. The cultures were subsequently washed twice with warm, unlabelled medium and then returned to humidified 95% air/5% CO_2 at 37°C and harvested at 0, 0.5, 1, 1.5, 2.0, and 4.0 h post-chase. The chromatin and NM-IF fractions were collected as described below.

Protein Extraction

Whole cell lysates were obtained by washing the adherent cells with PBS followed by scraping with extraction buffer (8M urea, 2% NP-40, 2% B-mercaptoethanol, 0.15 μM aprotinin, 1 mM benzamidine · HCl, 5.26 μM leupeptin, 1.52 μM pepstatin, and 1.2 mM PMSF). The lysate was clarified by brief centrifugation, snap frozen in liquid nitrogen and stored at −80°C. Cell fractions including the soluble and membrane-associated proteins, the cytoskeletal proteins, the intermediate filaments, chromatin proteins and the nuclear matrix proteins were obtained from a standard sequential extraction protocol [Fey et al., 1984]. Briefly, adherent cells were washed with ice cold PBS. Soluble and membrane proteins were extracted by adding CSK Buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES [pH 6.8], 3 mM $MgCl_2$, 1 mM EGTA, 1.2 mM (PMSF), 0.5% Triton X-100). This treatment was followed by extraction of the cytoskeletal proteins using RSB-Majik Buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris [pH 7.4], 1.2 mM PMSF, 1% Tween-40, 0.5% sodium deoxycholate). Cells were scraped and incubated in Digestion Buffer (50 mM NaCl, 300 mM sucrose, 10 mM PIPES [pH 6.8], 3 mM $MgCl_2$ 1 mM EGTA, 0.5% Triton X-100, 1.2 mM PMSF, 100 μg/ml DNaseI, 50 μg/ml RNA) at room temperature for 20 min. Chromatin proteins were extracted by the addition of ammonium sulfate to a concentration of 0.25 M. After centrifugation and removal of the supernatant (chromatin), the nuclear matrix-intermediate filament (NM-IF) complex was either frozen and used for analysis or further fractionated by disassembly (8 M urea, 20 mM MES [pH 6.6], 1 mM EGTA, 0.1 mM $MgCl_2$, 1.2 mM PMSF, and 1% 2-mercaptoethanol) and dialysis overnight at room temperature against Assembly Buffer (150 mM KCl, 25 mM imidiazole-HCl [pH 7.1], 5 mM $MgCl_2$, 0.125 mM EGTA, 2 mM dithiothreitol (DTT), 0.2 mM PMSF). These conditions promote reassembly of the intermediate filaments which were separated from the solubilized nuclear matrix proteins by ultracentrifugation. The nuclear matrix proteins were snap frozen and stored at −80°C; the intermediate filament proteins were re-solubilized in the disassembly buffer and stored at −20°C.

Protein Analysis

Protein extracts were analyzed by 1-D SDS/PAGE and the gels developed by fluorography or by Western blotting (21). Measurements of total protein were made using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). For immunoblotting, proteins were transferred onto a PVDF membrane (Millipore Corp., Bedford, MA) by the semi-dry method [Ausubel et al., 1987]. Blocking and antibody incubation conditions depended on the particular antibody (see Results). Immunoblots were developed using chemiluminescence (ECL™, Amersham).

Rigor Experiment

The ^{35}S-methionine-labelled nuclear matrix-intermediate filament (NM-IF) pellets from ROS 17/2.8 cells were resuspended in 250 μl of Rigor Buffer (75 mM KCl, 10 mM imidazole [pH 7.2], 2 mM EGTA, 2 mM $MgCl_2$, 1 mM NaN_3). To these suspensions, 10 μl of 100 mM ATP or water (control) was added. The control and ATP-treated suspensions were placed on ice for 15 min, then centrifuged. The supernatant was removed and 62.5 μl of glycerin was added (20% of total volume) before snap-freezing in liquid N_2 and storing at −80°C. Pellets were resuspended in 100 μl of 8M urea and stored at −20°C. These fractions were analyzed by 1-D SDS/PAGE and developed by fluorography [Ausubel et al., 1987].

Molecular Weight and Densitometry Analyses

Protein bands detected by ^{35}S-autoradiography, or antibody/chemiluminescence, were analyzed for intensity, area, and molecular weight using the gel documentation system Foto/analyst II and the software Collage™ (Fotodyne Inc., Hartland, WI). Images of protein signals were obtained with a CCD camera and stored as a TIFF file on an Apple MacIntosh Centris 650. The reported molecular weights of our unknown proteins are approximations based on migration against calibration standards and fitting the data to various regression models (linear, quadratic, cubic, logistic, and cubic spline).

Northern Analysis

Total cellular RNA from hormone-treated and control cells was isolated by lysing the cells with RNAzol™ B (CINNA/BIOTEX Laboratories, Inc. Houston, TX) and then extracting the lysate with chloroform. Aliquots of equal amounts of RNA (20 μg) were denatured and electrophoresed on a 1% agarose/6.6% formaldehyde gel and transferred onto a nylon membrane (Hybond-N™, Amersham). The RNA was cross-linked to the blot with UV light and the membrane prehybridized in Rapid-hyb Buffer (Amersham) for 2 h at 65°C. Hybridizations were performed using randomly primed [α-^{32}P]deoxy-CTP-labeled cDNA probes (SA > 1×10^9 dpm/μg) of rat PTH receptor. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the signals from these probes. The membranes were hybridized with the probes for 2 h at 70°C in the rapid-hyb Buffer. Subsequent to hybridization the blots were washed with 2 × SSC/0.1% SDS at room temperature for 1 h, followed by two 15 min washes with 0.2 × SSC/0.1% SDS at 65°C with a final wash with 2 × SSC at room temperature for 5 min. Blots were autoradiographed overnight at −80°C using Kodak X-AR film with intensifying screens.

RESULTS

Nuclear Matrix-Intermediate Filament Proteins of Osteoblasts and Osteosarcoma Cells

The 1-D SDS/PAGE profiles of ^{35}S-methionine-labelled nuclear matrix and nuclear matrix-intermediate filament proteins of confluent ROS 17/2.8 osteosarcoma cells and primary osteoblasts were similar (Fig. 1). The 200 and 190 kD, PTH-responsive proteins, originally described in ROS 17/2.8 cells [Bidwell et al., 1994b], were also present in the primary osteoblasts. Osteosarcoma cells and osteoblasts chronically labelled with ^{35}S-methionine for 24 h exhibited the 200 kD protein but not the 190 kD protein. The 200 kD protein was observed in the nuclear matrix-intermediate filament and intermediate filament fractions of our cell preparations but did not partition into the isolated nuclear matrix fractions of the osteosarcoma cells or osteoblasts, indicating that this protein was excluded from the nucleus. The 190 kD protein was recovered in the isolated nuclear matrix, intermediate filament, and nuclear matrix-intermediate filament fractions. Chronic PTH (72 h, 10 nM) attenuated the expression of the 200 kD protein to <50% of controls and downregulated the 190 kD protein several-fold in both cell preparations as measured by densitometry. In the osteoblasts, however, although the 190 kD band(s) recovered in the nuclear matrix fraction was downregulated by PTH, the 190 kD band(s) exhibited in the nuclear matrix-intermediate filament fraction was refractive to hormone exposure (Fig. 1B).

The PTH-responsive 160 kD protein, previously observed, in the nuclear matrix-intermediate filament and chromatin subfractions of the ROS 17/2.8 cells [Bidwell et al., 1994b], was detected in the isolated nuclear matrix fraction (Fig. 1A) but could not be clearly distinguished in the nuclear matrix-intermediate filament or nuclear matrix fractions of the osteoblasts (Fig. 1B). This protein band may have been obscured by other osteoblast protein bands in this fraction since the 160 kD protein was observed in the chromatin fraction of these cells (see below).

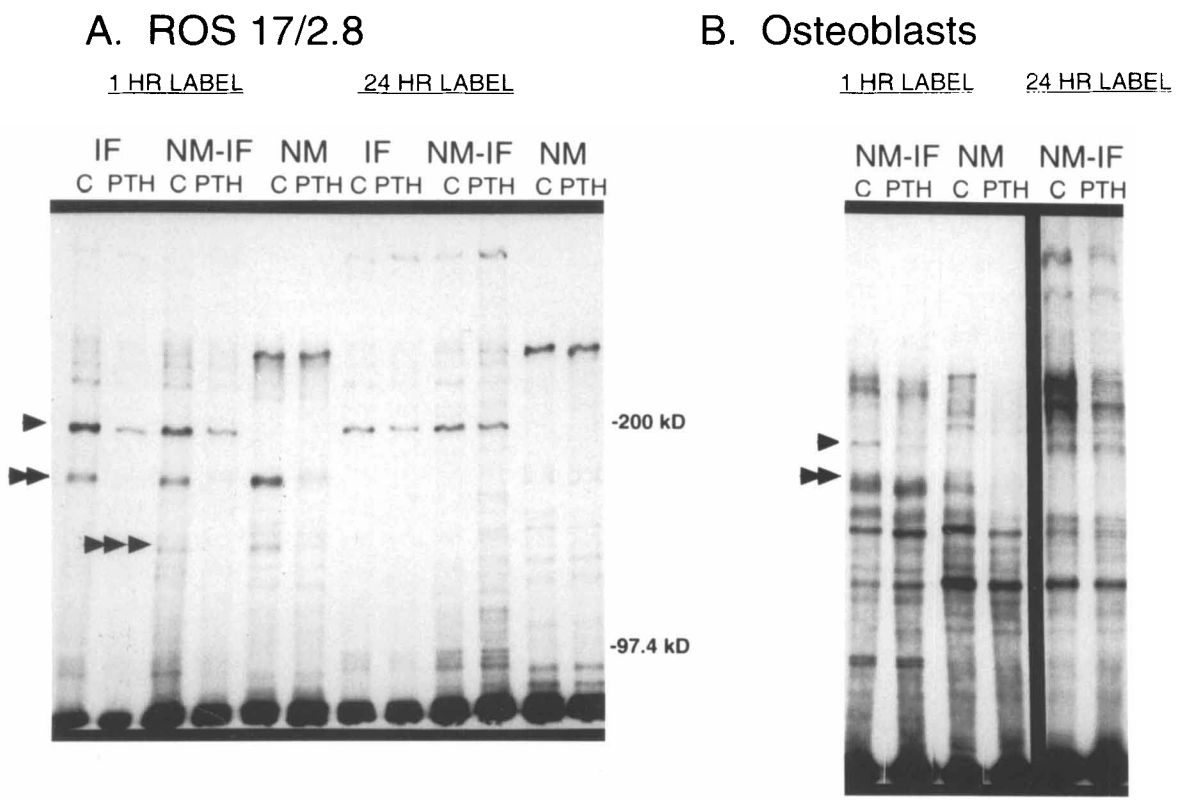

Fig. 1. 1-D SDS/PAGE (4% gel) profiles of ^{35}S-methionine-labelled, PTH-responsive nuclear matrix (NM), intermediate filament (IF), and NM-IF proteins of **(A)** ROS 17/2.8 osteosarcoma cells and **(B)** primary spongiosa osteoblasts [25,000 dpm/lane]. Cells were labelled 1 or 24 h prior to harvest (25 μCi/ml). The cells were treated with rat PTH[1-34] (10 nM) or the same volume of vehicle (10 mM acetic acid) for 3 successive 24 h cycles. The 200, 190, and 160 kD proteins are marked with single, double, and triple arrowheads, respectively.

The 160 kD protein was not observed in osteosarcoma cells or osteoblasts chronically labelled with ^{35}S-methionine for 24 h (Fig. 1). Consistent with the earlier study [Bidwell et al., 1994b], the 160 kD protein was downregulated several fold by chronic PTH exposure.

200 kD PTH-Responsive Protein

Based on the partitioning of the 200 kD protein into the intermediate filament fraction (Fig. 1A) we tested whether it was an ATP-dissociable protein, consistent with actin-binding proteins such as myosin. The nuclear matrix-intermediate filament pellet of the ROS 17/2.8 cells was resuspended in Rigor Buffer and either ATP (4 mM final concentration) or an equal volume of water was added. Following incubation (4°C) and centrifugation, the pellets and supernatants were analyzed 1-D SDS/PAGE. The addition of ATP induced the partitioning of the 200 kD protein and a protein of ~80 kD into the supernatant fraction (Fig. 2A). Western analysis of the nuclear matrix and intermediate filament fractions of ROS 17/2.8 cells, using an antibody to a nonmuscle myosin, resulted in a 200 kD band that resembled the subcellular distribution and PTH-responsiveness of the 200 kD protein band on the gels of the ^{35}S-methionine-labelled proteins (Fig. 2B). Western analysis of the nuclear matrix-intermediate filament extracts from the osteoblasts yielded similar results (Fig. 2B).

190 and 160 kD PTH-Responsive Proteins

The absence of the 190 and 160 kD protein bands in extracts from osteosarcoma cells and osteoblasts chronically labelled with ^{35}S-methionine for 24 h before harvest (Fig. 1) indicated these were low-abundant proteins with half-lives of less than 24 h. Since the 190 and 160 kD proteins were present in the chromatin fractions of both the osteosarcoma cells and osteoblasts, we used this larger and more concentrated fraction to further characterize the turnover of these proteins. Pulse-chase experiments revealed that both the 190 and 160 kD proteins had half-lives of approximately 1 h in

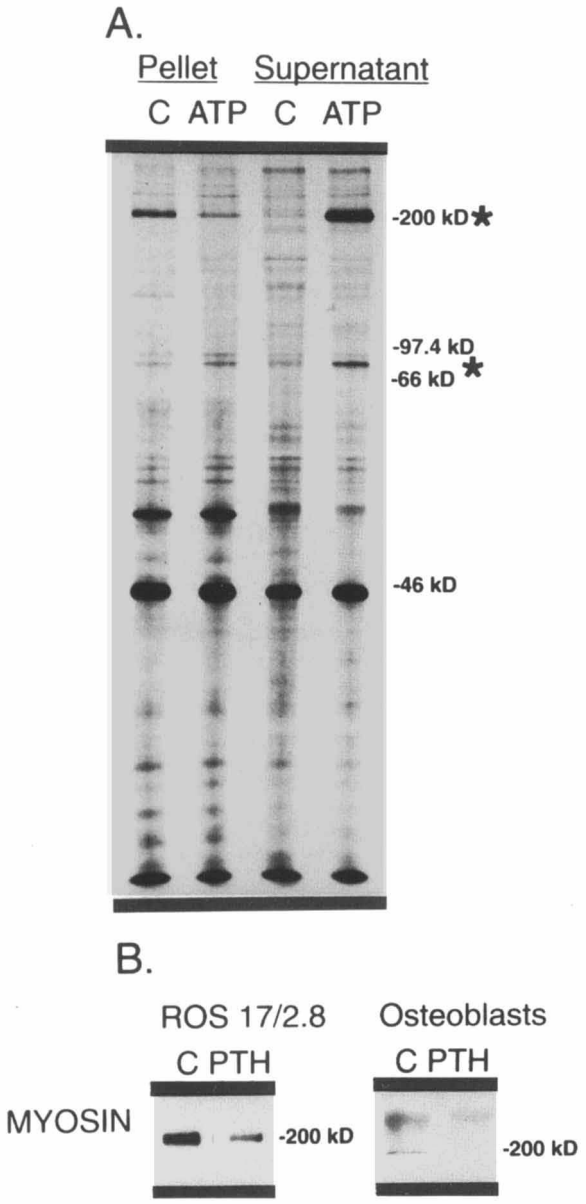

Fig. 2. **A:** Rigor experiment. ^{35}S-methionine-labelled nuclear matrix-intermediate filament pellets from ROS 17/2.8 cells were resuspended in 250 μl of Rigor Buffer. To these suspensions 10 μl of 100 mM ATP or water (control) was added. The control and ATP-treated suspensions were placed on ice for 15 min, then centrifuged. The pellet and supernatant fractions were electrophoresed on a 10%, low pH (8.6) gel and developed by fluorography. The 200 kD protein and an 80 kD protein are ATP-dissociable (marked by the asterisks). **B:** Western analysis of the subfractions of ROS 17/2.8 (intermediate filaments, 4 μg protein/lane) and rat osteoblasts (nuclear matrix-intermediate filament, 25 μg/lane) using a non-muscle myosin (1:5,000, primary antibody; 1:1,000, secondary antibody). Cells were treated with PTH as described in Figure 1A,B. Representative of three different experiments. PTH exposure attenuated the myosin antigenic signal ~ 50%, as measured by densitometry, in both the osteosarcoma cells and the osteoblasts, although this varied from 15–100% between cell cultures. No myosin was detected in the isolated nuclear matrix fractions (data not shown).

the ROS 17/2.8 cells (Fig 3A). The results of the experiments with the osteoblasts were more complex. Although no signal for these proteins were detected in chronically labelled cells, pulse-chase experiments yielded results indicating half-lives between ~ 1 h (Fig. 3B) and 4 h.

In both the osteosarcoma and osteoblast cell preparations we occasionally observed a protein band that migrated just below the 190 kD protein. Also, two protein bands were frequently observed at ~ 160 kD. Whether these bands represent variants of the same protein or different proteins is unclear.

Synthesis of the 190 and 160 kD proteins, determined by metabolic labelling of cells with ^{35}S-methionine for 1 h, was downregulated in response to acute hormone exposure (Fig 4). These protein bands were decreased to 50% of the control within the first hour of hormone exposure, as measured by densitometry (Fig. 4).

The molecular weights, subcellular distribution, and half-lives of the 190 and 160 kD proteins were similar to the nuclear matrix proteins topoisomerase II-α (170 kD), -β (180 kD), and an α-related isoform (160 kD) involved in mediating DNA topology [Heck et al., 1988]. To determine if these proteins were isoforms of topoisomerase II, we used Western analysis to probe chromatin fractions of ROS 17/2.8 osteosarcoma cells and the osteoblasts for topoisomerase II-α and -β. PTH exposure upregulated the expression of antigens of 170 and 160 kD in the ROS 17/2.8 cells by ~ 2-fold and 2–3-fold, respectively, as measured by densitometry (Fig. 5A). We could not consistently detect topoisomerase II-α in the osteoblasts by immunoblotting. Topoisomerase II-β expression was not modulated by PTH in either the osteosarcoma cells or the osteoblasts (Fig. 5B). These results indicated that neither the 190 nor 160 kD ^{35}S-methionine-labelled proteins cross-reacted with our antibodies to topoisomerase II.

Previously, we demonstrated that an antibody to NuMA, a 236 kD structural protein of the nuclear matrix, recognized a protein at ~ 220 kD in the whole cell lysates of human osteoblasts but revealed a band at ~ 200 kD in the nuclear matrix fraction of these cells [McCabe et al., 1996]. To determine if the 190 kD protein was immunoreactive with this antibody to NuMA, we performed Western analysis of whole cell lysates, nuclear matrix, intermediate filament, and chromatin fractions from ROS 17/2.8 cells and the osteoblasts. *Similar to our findings*

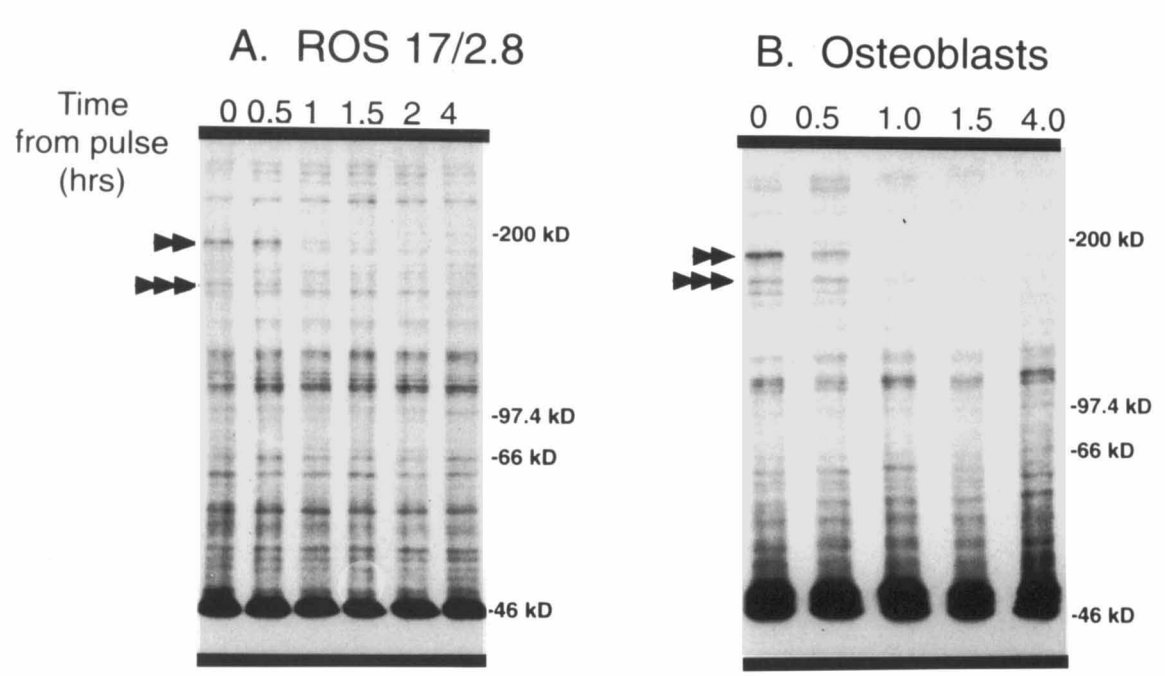

Fig. 3. Pulse-chase experiments for the 190 and 160 kD proteins. ROS 17/2.8 cells (**A**) and rat osteoblasts (**B**) were labelled with ^{35}S-methionine (25 μCi/ml) for 1 h, chased with unlabelled medium, and harvested at the time points indicated. Osteosarcoma and osteoblast chromatin fractions (25,000 and 75,000 dpm/lane, respectively) were electrophoresed on 5% gels and developed by fluorography. The 190 and 160 kD proteins are marked with double and triple arrowheads, respectively. The osteosarcoma results are representative of three different experiments. Although no signal for the 190 and 160 kD proteins were detected in chronically labelled osteoblasts, pusle-chase experiments yielded results indicating half-lives between ~1 h (shown here) and 4 h.

with the human osteoblasts, this antibody recognized a protein band at ~220 kD in the whole cell lysates and a doublet at ~200 kD in the nuclear matrix fraction of the ROS 17/2.8 cells (Fig. 5C). NuMA was also recovered in the intermediate filament and chromatin fractions. Exposure to PTH upregulated the expression of NuMA in the ROS 17/2.8 cells by ~2-fold as determined by densitometry. We could not consistently detect NuMA in the osteoblasts by immunoblotting. Finally, PTH did not modulate the expression of β-actin in either the ROS 17/2.8 cells (Fig. 5D) or the osteoblasts (data not shown).

Consistent with an identity distinct from topoisomerase II or NuMA, the 190 kD protein exhibited a more limited tissue distribution as compared to the nearly ubiquitous expression of these known nuclear matrix proteins [Juenke and Holden, 1993; Zeng et al., 1994]. The 190 kD protein was not observed in the chromatin or nuclear matrix-intermediate filament fractions of H4 rat hepatoma cells, NIH3T3 mouse fibroblasts, or OK opossum kidney cells (Fig. 6). We did detect, however, a protein band in the NM-IF fractions of the H4 and NIH3T3 cells that migrated just below the 190 kD protein (Fig. 6B). The 160 kD protein was observed in the nuclear matrix-intermediate fractions of the H4 and the NIH3T3 cells.

PTH Receptor

To confirm that PTH regulated the expression of membrane proteins in our cell preparations we performed Northern analysis for PTH receptor mRNA in cells treated with hormone or vehicle. In confluent ROS 17/2.8 cells and osteoblasts, exposure to rPTH (1-34) (72 h, 10 nM) downregulated receptor mRNA expression by ~60–70% of controls as measured by densitometry (Fig. 7).

DISCUSSION

PTH selectively regulated the expression of membrane, cytoskeletal, and nuclear matrix proteins in both rat osteosarcoma cells and osteoblasts derived from the primary spongiosa of the metaphyseal distal femur of young rats. These data suggest that PTH coordinately integrates changes in osteoblast architecture through the tissue matrix, culminating in specific alterations in nuclear structure.

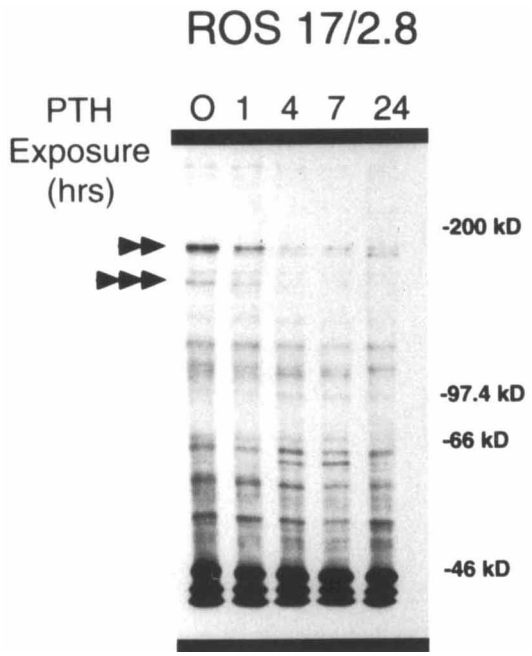

Fig. 4. The 190 and 160 kD protein response to acute PTH exposure. ROS 17/2.8 cells were exposed to rat PTH(1-34) [10 nM] for the times indicated and labelled for 1 h before harvest as described in Figure 1. Chromatin fractions, 25,000 dpm/lane (5% gel). The 190 and 160 kD proteins are marked with double and triple arrowheads, respectively. The synthesis of these proteins was attenuated by 50% within the first hour of hormone exposure as measured by densitometry. Note that there is a band just below the 190 kD protein band that is more clearly seen after a few hours of PTH exposure.

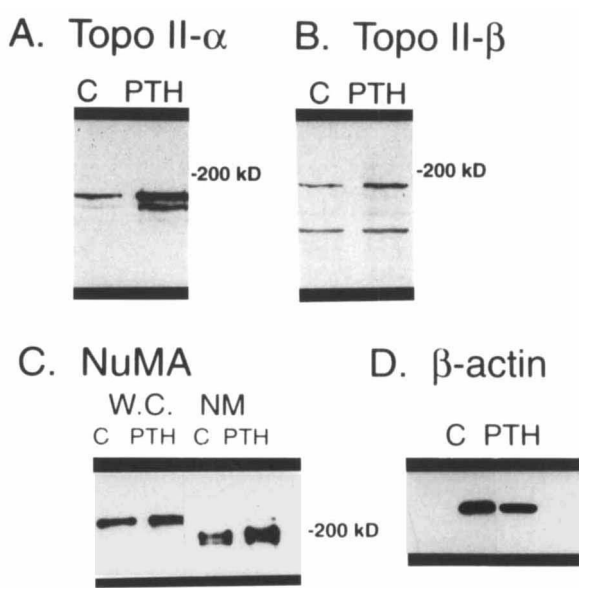

Fig. 5. Western analyses of ROS 17/2.8 cellular subfractions from cells treated with PTH or vehicle as described in Figure 1. **A:** Analysis for topoisomerase II-α in chromatin fraction, 50 μg protein/lane (1:1,000 primary and secondary antibodies). Treatment with PTH resulted in 2-fold increase in the top band and ~2–3-fold increase in the lower band as measured by densitometry. The top band [~170 kD] is topo II-α (as determined by a yeast positive control, data not shown), the second band may be topo II-α-related protein [~160 kD]. **B:** Analysis for topo II-β in chromatin fraction, 50 μg protein/lane (1:500 primary antibody, 1:15,000 secondary antibody). Two bands observed at ~180 and ~100 kD. PTH had no effect on the expression of this protein. **C:** Analysis for NuMA in whole cell lysates [W.C., 25 μg protein/lane] and the nuclear matrix fractions [NM, 10 μg protein/lane] (1:1,000 primary and secondary antibodies). The antigen in the whole cell lysates exhibited a molecular weight of ~220 kD whereas the antigen in the NM fraction was ~200 kD. PTH upregulated expression of NuMA by ~2-fold as measured by densitometry. **D:** Analysis for β-actin in whole cell lysates, 10 μg protein/lane (1:100,000 primary antibody, 1:1,000 secondary antibody). PTH exposure did not modulate β-actin expression. All blots are representative of three different experiments.

The 200 kD protein was identified as a non-muscle myosin. This is consistent with the molecular weight of the protein, its exclusion from the isolated nuclear fraction, its dissociation from the nuclear matrix-intermediate filament insoluble pellet with ATP, and Western analysis. Subtle differences in cell density between our cultures may have caused the variability in the magnitude of the PTH-induced decrease in myosin since cell density affects myosin and actin expression in cultured osteoblasts [Egan et al., 1991b].

PTH induced a decrease in myosin, in the Triton-X insoluble pellet of fetal rat calvarial osteoblasts, within 5 min of exposure [Egan et al., 1991a]. Myosin began returning to control levels at 10 min but remained below pretreatment levels for at least 6 h [Egan et al., 1991a]. No PTH-induced downregulation of myosin was observed in the ROS 17/2.8 cells at 8 h of hormone exposure [Bidwell et al., 1994a] suggesting that PTH can effect both transient and stable changes in myosin organization. Whether acute and chronic exposure to hormone results in distinct myosin architectures mediating different changes in gene expression is not known.

Although the 190 and 160 kD proteins were not topoisomerase II or NuMA, that PTH regulated expression of topoisomerase II-α is significant because it couples PTH-induced changes in cell shape to alterations in DNA topology. Topoisomerases organize DNA into cell type- and phenotype-specific loop domains which contribute to gene expression, including the specificity of cell response to a given hormone [Pienta et al., 1991]. These enzymes are involved in transcription, replication, recombination, and chro-

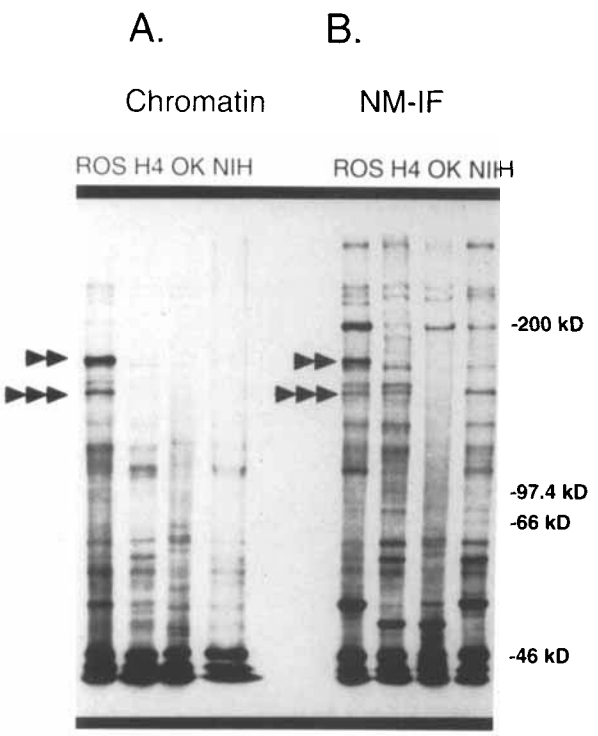

Fig 6. 1-D SDS/PAGE (5%) of labelled (**A**) chromatin and (**B**) NM-IF fractions of ROS 17/2.8 osteosarcoma cells, OK opossum kidney cells, NIH3T3 mouse fibroblasts, and H4 rat hepatoma cells. Cells were labelled 1 h prior to harvest as described in Figure 1. 25,000 dpm/lane. The 190 and 160 kD proteins are marked with double and triple arrowheads, respectively. Representative of two experiments.

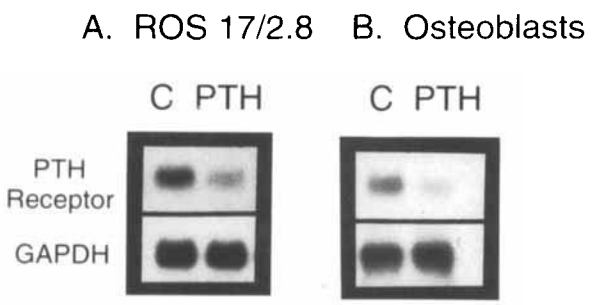

Fig. 7. Northern analysis for rat PTH receptor and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in (**A**) ROS 17/2.8 osteosarcoma cells and (**B**) rat osteoblasts. Cells were treated with PTH or vehicle as described in Figure 1. Exposure to hormone downregulated receptor mRNA expression by ~60–70% of controls as measured by densitometry; 20 μg RNA/lane. Representative of three experiments.

mosome segregation [Wigley, 1995]. Topoisomerase II-α and -β are most highly expressed in rapidly proliferating cells and cells that have reached the plateau phase of growth, respectively [Wigley, 1995; Giaccone, 1994]. Recent evidence indicates that the β-isoform is involved in terminal differentiation [Watanabe et al., 1994; Capranico et al., 1992]. Thus, topoisomerase II enzymes may mediate the transition from a nuclear architecture supporting proliferation to one sustaining differentiation.

Similarly, PTH regulation of NuMA expression may alter osteoblast nuclear architecture. NuMA, a component of the nuclear core filaments, plays a structural role in nuclear organization during interphase but organizes the microtubules of the mitotic spindle during mitogenesis [Zeng et al., 1994]. NuMA contains p34cdc2 phosphorylation sites that mediate association with the mitotic spindle [Compton and Luo, 1995] and it may have been different phosphorylation states of this protein, induced during extraction, that caused the shift in NuMA mobility on our Western blots.

The functional significance of the 190 and 160 kD proteins remains to be established. The 190 kD protein may be an isoform of topoisomerase II not recognized by our antibody, perhaps representing an osteoblast-specific form of the enzyme. A B-cell-specific, topoisomerase II-related protein may act to modulate torsional stress in the immunoglobulin μ heavy chain promoter [Webb et al., 1993]. The 190 and 160 kD proteins may be cell cycle-regulated in osteoblasts and not in the osteosarcoma cells, consistent with the variable pulse-chase results with the primary spongiosa cells. The subfraction distribution of these proteins indicate they may either shuttle between the nuclear scaffold and chromatin or be ribonuclear proteins released from the nuclear matrix by RNAse A digestion. That the 190 kD band in the osteoblast nuclear matrix-intermediate filament fraction was refractive to PTH suggests that the hormone induced a redistribution of this protein from the nucleus to the perinuclear space. Alternatively, there may be multiple 190 kD proteins in our cell fractions.

These data demonstrate that PTH-induced downregulation involves a reorganization within the osteoblast nucleus. This may include a lockdown of target genes preventing transcriptional activation [Bodnar et al., 1989]. Also, the response of the 190 and 160 kD proteins to acute hormone exposure indicates that PTH-induced changes in osteoblast nuclear structure can occur rapidly, consistent with rapid cytoskeletal reorganization [Egan et al., 1991a].

The integration of PTH-induced mobilization of second messengers and changes in osteoblast

shape comprise the signal transduction cascade responsible for changes in gene expression. Topoisomerase II, NuMA, and the 190 and 160 kD proteins, may direct the nuclear PTH signalling pathways to the genes and mediate the structural elements of the transcriptional response.

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REFERENCES

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl D (eds) (1987): "Current Protocols in Molecular Biology." New York: John Wiley & Sons, Inc.

Berezney R (1991): The nuclear matrix: A heuristic model for investigating genomic organization and function in the cell nucleus. J Cell Biochem 47:109–123.

Berezney R, Coffey DS (1975): Nuclear protein matrix: Association with newly synthesized DNA. Science 189:291–292.

Bidwell JP, Fey EG, van Wijnen AJ, Penman S, Stein JL, Lian JB, Stein GS (1994a): Nuclear matrix proteins distinguish normal diploid osteoblasts from osteosarcoma cells. Cancer Res 54:28–32.

Bidwell J, van Wijnen A, Banerjee C, Fey E, Merriman H, Penman S, Stein J, Lian J, Stein G (1994b): Parathyroid-responsive modifications in the nuclear matrix of ROS 17/2.8 rat osteosarcoma cells. Endocrinology 134:1738–1744.

Bodnar JW, Jones GS, Ellis CH Jr (1989): The domain model for eukaryotic DNA organization. 2: A molecular basis for constraints on development and evolution. J Theor Biol 137:281–320.

Capranico G, Tinelli S, Austin CA, Fisher ML, Zunino F (1992): Different patterns of gene expression of topoiosomerase II isoforms in differentiated tissues during murine development. Biochim Biophys Acta 1132:43–48.

Carter KC, Bowman D, Carrington W, Fogarty K, McNeil JA, Fay FS, Lawrence JB (1993): A three-dimensional view of precursor messenger RNA metabolism within the mammalian nucleus. Science 259:1330–1335.

Compton DA, Luo C (1995): Mutation of the predicted p34cdc2 phosphorylation sites in NuMa impair the assembly of the mitotic spindle and block mitosis. J Cell Sci 108:621–633.

Dworetzky SI, Fey EG, Penman S, Lian JB, Stein JL, Stein GS (1990): Progressive changes in the protein composition of the nuclear matrix during rat osteoblast differentiation. Proc Natl Acad Sci USA 87:4605–4609.

Egan JJ, Gronowicz G, Rodan GA (1991a): Parathyroid hormone promotes the disassembly of cytoskeletal actin and myosin in cultured osteoblastic cells: Mediation by cyclic AMP. J Cell Biochem 45:101–111.

Egan JJ, Gronowicz G, Rodan GA (1991b): Cell density-dependent decrease in cytoskeletal actin and myosin in cultured osteoblastic cells: Correlation with cyclic AMP changes. J Cell Biochem 45:93–100.

Fey EG, Wan J, Penman S (1984): The epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: Three-dimensional organization and protein composition. J Cell Biol 19:1973–1984.

Getzenberg RH, Pienta KJ, Coffey DS (1990): The tissue matrix: Cell dynamics and hormone action. Endocrine Rev 11:399–413.

Giaccone G (1994): DNA topoisomerases and topoisomerase inhibitors. Path Biol 42:346–352.

Heck MM, Hittelman WN, Earnshaw WC (1988): Differential expression of DNA topoisomerase I and II during the eukaryotic cell cycle. Proc Natl Acad Sci USA 85:1086–1090.

Juenke JM, Holden JA (1993): The distribution of DNA topoisomerase II isoforms in differentiated adult mouse tissues. Biochim Biophys Acta 1216:191–196.

Long H, Onyia J, Hulman J, Bidwell J, Hock JM (1995): Molecular characterization of primary spongiosa cells from distal femurs of young rats. J Bone Min Res 10:S312 (abst).

Lormi A, Marie PJ (1990): Changes in cytoskeletal proteins in response to parathyroid hormone and 1,25-dihydroxyvitamin D in human osteoblastic cells. Bone Miner 10:1–12.

McCabe R, Feister H, Hock JM, Bidwell JP (1996): Nuclear matrix and intermediate filament proteins of a human osteosarcoma tumor. J Dent Res 75:278 (abstr).

Merriman HL, van Wijnen AJ, Hiebert S, Bidwell JP, Fey E, Lian J, Stein J, Stein GS (1995): The tissue-specific nuclear protein, NMP-2, is a member of the AML/CBF/PEBP2/runt domain transcription factor family: Interactions with the osteocalcin gene promoter. Biochemistry 34:13125–32.

Mirkovitch J, Mirault M-E, Laemmli UK (1984): Organization of the higher order chromatin loop: Specific DNA attachment sites on nuclear scaffold. Cell 39:223–232.

Nakagomi K, Kohwi Y, Dickinson LA, Kohwi-Shigematsu T (1994): A novel DNA-binding motif in the nuclear matrix attachment DNA-binding protein SATB1. Mol Cell Biol 14:1852–60.

Nickerson JA, Penman S (1992): Localization of nuclear matrix core filament proteins at interphase and mitosis. Cell Bio Int Rep 16:811–826.

Nissenson RA, Huang Z, Blind E, Shoback D (1993): Structure and function of the receptor for parathyroid hormone and parathyroid hormone-related protein. Receptor 3:193–202.

Partridge NC, Bloch SR, Pearman AT (1994): Signal transduction pathways mediating parathyroid hormone regulation of osteoblastic gene expression. J Cell Biochem 55:321–327.

Pienta KJ, Getzenberg RH, Coffey DH (1991): Cell structure and DNA organization. Eukaryotic Gene Expression 1:355–385.

Razin SV, Gromova II (1995): The channels model of nuclear matrix structure. Bioessays 17:443–450.

Sato M, Rodan GA (1989): Bone cell shape and function. In Stein WD, Branner F (eds): "Cell Shape: Determinants, Regulation, and Regulatory Role." San Diego, CA: Academic Press, Inc.

Watanbe M, Tsutsui K, Inoue Y (1994): Differential expressions of the topoisomerase II alpha and II beta mRNAs in developing rat brain. Neurosci Res 19:51–57.

Webb CF, Eneff KL, Drake FH (1993): A topoisomerase II-like protein is part of an inducible DNA-binding protein complex that binds 5′ of an immunoglobin promoter. Nucleic Acids Res 21:4363–4368.

Wigley DB (1995): Structure and mechanism of DNA topoisomerases. Annu Rev Biophys Biomol Struct 24:185–208.

Zeng C, He D, Brinkley BR (1994): Localization of NuMA protein isoforms in the nuclear matrix of mammalian cells. Cell Motil Cytoskeleton 29:167–176.