Regulation of Bone Sialoprotein mRNA by Steroid Hormones

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Abstract. In this report we demonstrate an increase in the steady-state level of bone sialoprotein (BSP) mRNA in rat calvaria and a rat osteosarcoma cell line (ROS 17/2.8) after treatment with the synthetic glucocorticoid, dexamethasone. In contrast, 1.25-dihydroxyvitamin D_3 reduced the amount of BSP mRNA in calvaria and inhibited the dexamethasone induction in ROS 17/2.8 cells. The increase in BSP mRNA is most likely due to an increase in the transcriptional rate. The stability of mRNA was unchanged after dexamethasone treatment with a half-life of ~5 h. Nuclear transcription experiments with nuclei isolated from ROS 17/2.8 cells showed an increased BSP mRNA synthesis in cells treated with dexamethasone.

BONE sialoprotein (BSP),[1] which we previously called sialoprotein II, is an acidic glycoprotein associated with the mineral matrix in bone and teeth (2, 3). The protein contains ~50% carbohydrate of which 15% is sialic acid (3). Furthermore, some 30% of the serine residues are phosphorylated and contribute to the acidity of BSP. The amino acid sequence of BSP, deduced from cDNA, predicts a 34-kD protein core with predominantly glutamic acid and glycine residues, which constitute one third of all amino acid residues (10). The protein contains several clusters of up to 10 consecutive glutamic acid residues. These negatively charged domains, together with the sialic acid residues and phosphate groups, are presumably responsible for the strong interaction with hydroxyapatite.

BSP promotes the attachment and spreading of rat osteosarcoma cells (ROS 17/12.8) as well as primary bovine chondrocytes in cell-binding experiments using plastic dishes coated with the protein (11, 12). This cell binding is apparently mediated by an RGD-containing region in BSP, which is homologous to the cell-binding domain in vitronectin (10). The BSP receptor is an integrin indistinguishable from the vitronectin receptor on the surface of ROS 17/2.8 cells (11).

BSP shares some structural features with another bone protein, osteopontin (OPN) (9). The OPN sequence contains a stretch of nine consecutive aspartic acid residues comparable to the glutamic acid clusters in BSP. OPN also contains a cell-binding RGD sequence which promotes attachment of cells in a similar way as BSP (9). The synthesis of OPN in bone cells is regulated by steroid hormones. It has been shown that 1,25-dihydroxyvitamin D_3 (vit D_3) increases the level of OPN mRNA, whereas the synthetic glucocorticoid, dexamethasone, reduces the steady-state level of OPN mRNA (15).

It is well established that steroid hormones have pronounced influence on the metabolism of bone. Some of these effects may be exerted via effects on cells, possibly mediated via cell-binding proteins. The present study, therefore, was undertaken to determine the effects of different steroid hormones on the biosynthesis of two major cell-binding bone proteins.

1. *Abbreviations used in this paper*: BSP, bone sialoprotein; DRB, 5,6-dichloro-1-β-ribofuranosyl benzimidazole; OPN, osteopontin; ROS, rat osteosarcoma; vit D_3, 1,25-dihydroxyvitamin D_3.

Materials and Methods

Cell and Tissue Cultures

Calvaria tissue was dissected from neonatal (3–4-d-old) rats. Isolated calvaria and the rat osteosarcoma cell line ROS 17/2.8 (6), respectively, were cultured in Ham's F12 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS. Dexamethasone (Sigma Chemical Co., St. Louis, MO) and vit D_3 (a gift from Sandoz, A. G., Basel) were added to culture media supplemented with 2% FCS. Both steroids were used at 10^{-8} M in all experiments. 5,6-Dichloro-1-β-ribofuranosyl benzimidazole (DRB) (Sigma Chemical Co., St. Louis, MO), a potent inhibitor of RNA polymerase II (16), was used at a concentration of 10^{-4} M.

Isolation and Analysis of RNA

RNA was isolated by extraction with guanidine isothiocyanate and centrifugation in a cesium chloride gradient (7). Electrophoresis and transfer of RNA to nitrocellulose were performed as previously described (9). The filters were hybridized with an OPN cDNA probe as described (9). The BSP cDNA probe represented a 446-bp Eco RI–PVU II fragment (nucleotide 1-446 in reference 10). Filters were hybridized with the BSP probe at 47°C in a hybridization solution containing 50% formamide (9). The filters were washed with 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at room temperature for 1 h and finally in 0.2 × SSC at 68°C for 1 h.

In Vitro Transcription Analysis

Nuclei were isolated from ROS 17/2.8 cells (3a) and transcribed in vitro (5) using the modifications described by Jäck and Wabl (4). ^{32}P-labeled RNA (2×10^8 cpm/ml) was hybridized to nitrocellulose filters carrying single-stranded BSP or OPN cDNA. An Eco RI–Pvu II fragment (nucleotide 1-446 in reference 10) was ligated to M13 mp18 and mp19 in sense and antisense orientation. Also an Eco RI fragment of λROP1 (9) was ligated to M13 in both orientations. Single-stranded M13 DNA was isolated and bound to nitrocellulose using a slot blot apparatus (Schleicher & Schuell, Inc., Keene, NH), 200 μg/well. The filters were hybridized with ^{32}P-

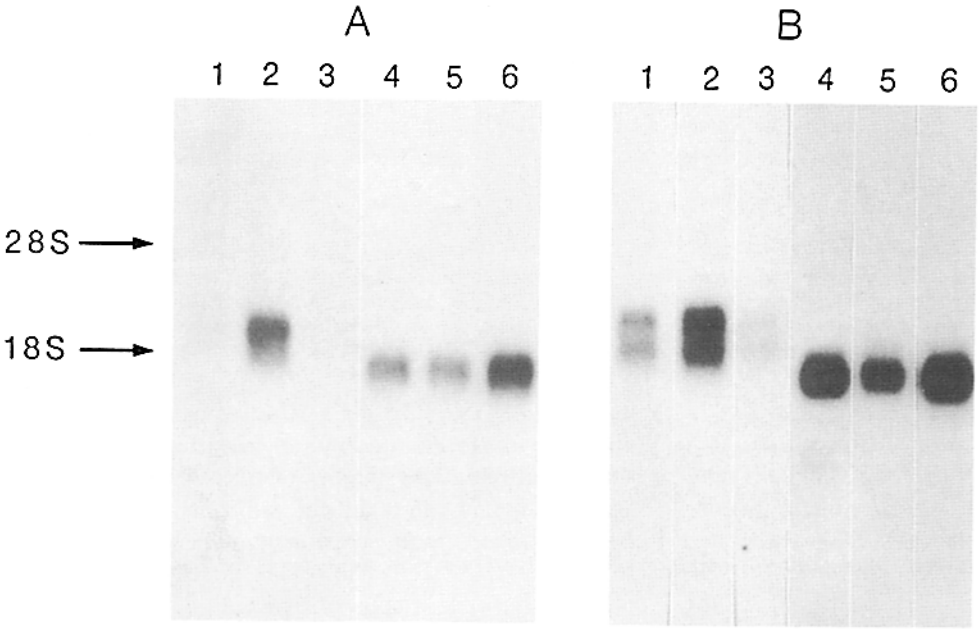

Figure 1. Transfer blot analysis of RNA (15 μg/lane) isolated from ROS 17/2.8 cells (*A*) and neonatal rat calvaria (*B*) cultured in medium with or without 10 nM steroid for 24 h. Lanes *1–3* were hybridized with a BSP cDNA probe and lanes *4–6* were hybridized with an OPN cDNA probe. (Lanes *1* and *4*) Controls without steroid; (lanes *2* and *5*) dexamethasone treated; (lanes *3* and *6*) vit D_3 treated.

labeled RNA and washed using the same conditions as described above for hybridization with BSP cDNA in RNA transfer blot analysis.

Results

We determined the effects of dexamethasone and vit D_3 on the steady-state levels of BSP mRNA in cultured neonatal rat calvaria and in the rat osteosarcoma cell line ROS 17/2.8 (Fig. 1). BSP mRNA levels increased threefold in calvaria tissue after exposure to dexamethasone for 24 h. In contrast, treatment with vit D_3 reduced the amount to one third of that in control without steroid. Similar effects of the steroids were seen in ROS 17/2.8 cells. Without dexamethasone or with vit D_3 no BSP mRNA was detected. Dexamethasone increased the amounts of BSP mRNA to detectable levels in cultured osteosarcoma cells. In control experiments an identical set of filters were hybridized with an OPN cDNA probe. Vit D_3 increased the steady-state level of OPN mRNA about fivefold in both calvaria and ROS 17/2.8 cells. This agrees with previous results reported by Yoon et al. (15). These authors also observed a reduction in OPN mRNA after treatment with dexamethasone. In the present set of experiments, however, we did not observe significantly decreased amounts of OPN mRNA after dexamethasone treatment. This may be due to a shorter dexamethasone exposure time of 24 h used by us as compared with 72 h used by Yoon et al. The amount of BSP mRNA increased with time in dexamethasone-stimulated ROS 17/2.8 cells (Fig. 2), although the amount of BSP mRNA fluctuated with time in some experiments. The amounts of BSP mRNA were, however, always above the amount in untreated cells. The reason for this variation in BSP mRNA levels is unknown. No BSP mRNA was detected when dexamethasone and vit D_3 were added simultaneously to the osteosarcoma cells. Apparently, the vit D_3 suppression of the BSP mRNA steady-state level overrides the stimulatory effect of dexamethasone (Fig. 2).

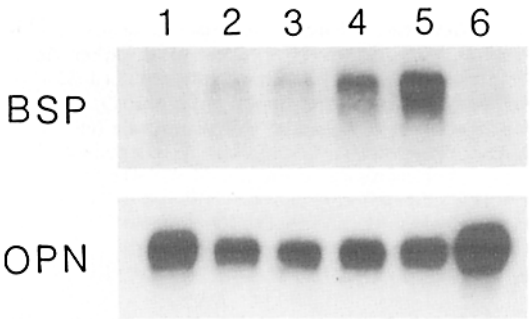

Figure 2. Time course of BSP mRNA increase in ROS 17/2.8 cells cultured in the presence of 10 nM dexamethasone. RNA (15 μg/lane) was separated on an agarose gel, transferred to nitrocellulose, and hybridized with a BSP cDNA probe (*top*) and an OPN cDNA probe (*bottom*). (Lane *1*) Control without dexamethasone; (lanes *2–5*) cells exposed to dexamethasone for 3, 6, 12, and 24 h (lane *6*) cells cultured in medium containing both dexamethasone (10 nM) and vit D_3 (10 nM) for 24 h.

To determine if the increase of BSP mRNA was due to an increased stability and half-life of mRNA we studied its degradation in calvaria tissue cultures (Fig. 3). Calvaria were kept in culture medium with or without dexamethasone for 24 h. After addition of DRB, a potent inhibitor of RNA synthesis (16), the rate of decreasing BSP mRNA was determined. The half-life of BSP mRNA was found to be 4–5 h and was not affected by added dexamethasone. This result indicated that the regulation of BSP mRNA by dexamethasone is at the transcriptional level. A corresponding half-life of 6 h was determined when the same filter was hybridized with the OPN cDNA probe (data not shown).

To provide further support for the assumption that the dexamethasone regulation is at the transcriptional level, we determined the amount of BSP mRNA synthesized by ROS 17/2.8 cells with or without dexamethasone in the culture medium (Fig. 4). Radiolabeled RNA was synthesized in vitro in isolated nuclei and hybridized to filters carrying the single-stranded M13 vector with BSP or OPN cDNA, in sense or antisense orientation. Labeled RNA from nuclei of dexamethasone exposed cells hybridized to single-stranded

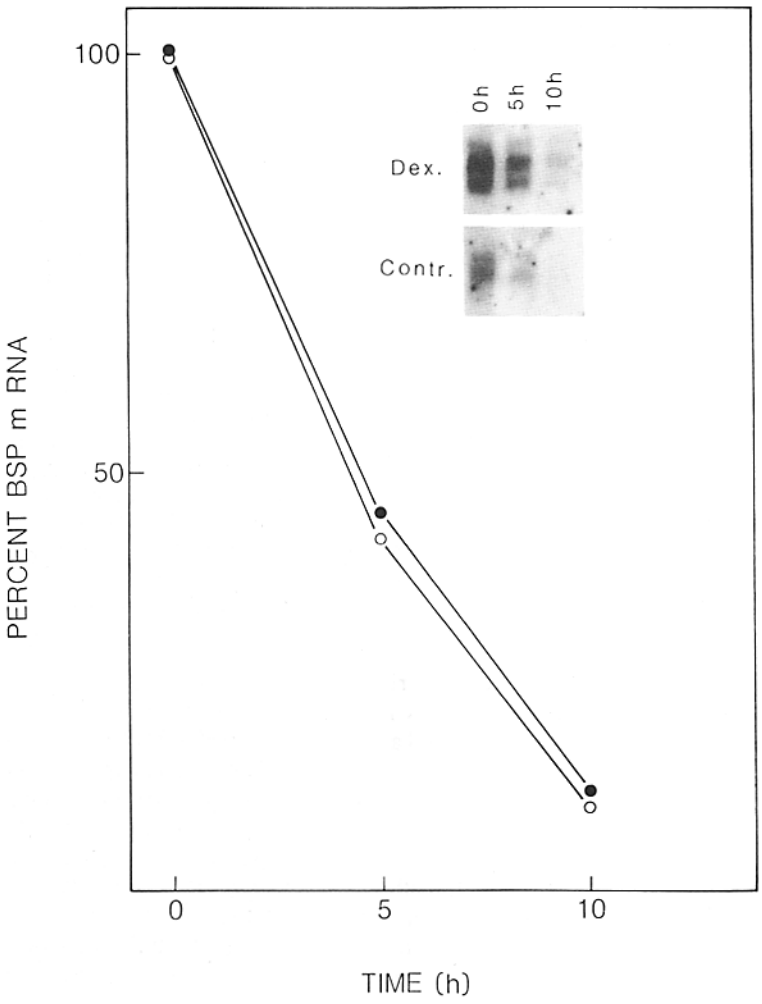

Figure 3. Degradation of BSP mRNA in calvaria. Calvaria were cultured in medium with (○) or without (●) 10 nM dexamethasone for 24 h. DRB was added to a final concentration of 100 nM. RNA was isolated after 5 and 10 h and subjected to transfer blot analysis with BSP cDNA as a probe. The amount of BSP mRNA before addition of DRB was set to 100%. The blots used for densitometric scanning are shown inserted.

BSP cDNA in the antisense but not in the sense orientation. No hybridization with BSP was seen with RNA from untreated nuclei. The control indicates that similar amounts of OPN mRNA were synthesized with and without dexamethasone. This nuclear run on experiment shows that dexamethasone increases the transcriptional rate of BSP mRNA. A separate experiment was set up with ROS 17/2.8 cells to study the role of active protein synthesis in the increased transcription of BSP mRNA upon dexamethasone treatment. Thus, cycloheximide when added simultaneously with the steroid abolished its effect on the BSP mRNA synthesis (Fig. 5).

Discussion

The physiological significance of glucocorticoids in bone homeostasis is unclear but the long-term therapeutic effect on patients is a decrease in bone mass. In model systems of in vitro bone formation, however, dexamethasone has been demonstrated to promote the formation of calcified bone nodules by primary cells isolated from rat calvaria (1). This has been proposed to be due to a stimulatory effect of dexamethasone on the maturation of determined osteoprogenitor cells. It has also been suggested that the decrease in bone

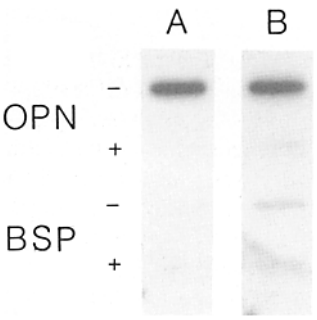

Figure 4. Nuclear transcription assay of nuclei isolated from ROS 17/2.8 cells cultured without (*A*) or with (*B*) 10 nM dexamethasone for 24 h. ^{32}P-labeled RNA transcribed in vitro was hybridized with nitrocellulose filters carrying single-stranded M13 vector with BSP or OPN cDNA in antisense (−) or sense (+) orientation.

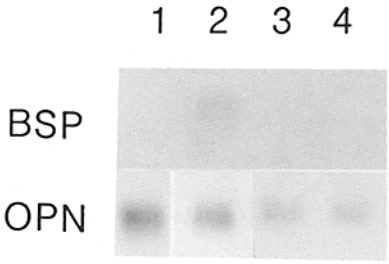

Figure 5. Effect of cycloheximide on the induction of BSP mRNA by dexamethasone. ROS 17/2.8 cells were cultured for 10 h in medium (lane *1*) without additive, (lane *2*) containing 10 nM dexamethasone, (lane *3*) containing 5×10^{-5} M cycloheximide, or (lane *4*) containing 10 nM dexamethasone plus 5×10^{-5} M cycloheximide. Total RNA was isolated from the cells and 15 μg was analyzed in transfer blot analysis with BSP (*top*) or OPN (*bottom*) as probes.

mass observed after long-term use of glucocorticoids in therapy follows from a depletion of the reserves of preosteoblasts and consequently a decrease in the number of bone mineral-producing osteoblasts (13). In this context it is relevant to note that the ROS 17/2.8 cell line attains a more osteoblastic phenotype in the presence of dexamethasone (14). The glucocorticoid causes the osteosarcoma cells to increase their expression of alkaline phosphatase, a key enzyme in the formation of hydroxyapatite. Interestingly, the ROS 17/2.8 cells when cultured with dexamethasone grow to a lower density and are morphologically different from cells in the absence of dexamethasone (14). The more adherent and flattened morphology induced by the corticosteroid may be a result of increased synthesis of BSP, which promotes attachment and spreading of ROS 17/2.8 cells.

The exact mechanism of dexamethasone action on the transcription of BSP mRNA is unclear. The comparatively long time needed to reach maximum levels of BSP mRNA in ROS 17/2.8 cells indicates that it does not involve the classical binding to enhancer elements in the BSP gene of the glucocorticoid hormone–glucocorticoid receptor complex. Furthermore, we have observed that the increase in BSP mRNA requires active protein synthesis, since cycloheximide prevented the effect. No change in the morphology of the cells was observed during the incubation period, indicating that the abolished dexamethasone effect was not due to acute toxicity of cycloheximide. Cycloheximide treatment gave some reduction of the expression of OPN mRNA, probably a result of the decreasing number of viable cells during the experiment. The loss of expression of BSP mRNA during these conditions, however, indicates a more pronounced effect on the expression of this protein. Thus, the effect of dexamethasone on the steady-state level of BSP mRNA is most likely indirect and may involve changes in the phenotype of the osteosarcoma cells. A corresponding effect on bone tissue, e.g., calvaria, could involve maturation of preosteoblasts. In support, the synthesis of BSP mRNA in both calvaria and ROS 17/2.8 cells was inhibited by vit D_3, which has been proposed to inhibit the maturation of osteoblasts from progenitor cells (Ishida, H., D. G. Bellows, J. E. Aubin, and J. N. M. Heersche, *Calcif. Tissue Int.*, Suppl. 42, Abstr. 19). This effect is also in accordance with the induced mobilization of calcium into the circulation by vit D_3.

The effects of steroids on the synthesis of BSP mRNA suggest that the protein is a product of mature osteoblasts producing bone matrix. The function of the protein is unknown but it may be associated with key functions of bone-producing cells, such as regulation of hydroxyapatite crystal nucleation and growth.

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