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Transforming Growth Factor-β1 Regulation of Bone Sialoprotein Gene Transcription: Identification of a TGF-β Activation Element in the Rat BSP Gene Promoter

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Abstract Transforming growth factor-β (TGF-β) increases steady-state mRNA levels of several extracellular matrix proteins in mineralized connective tissues. Bone sialoprotein (BSP) is a major constituent of the bone matrix, thought to initiate and regulate the formation of mineral crystals. To determine the molecular pathways of TGF-β1 regulation of bone proteins, we have analyzed the effects of the TGF-β1 on the expression of the BSP in the rat osteosarcoma cell line (ROS 17/2.8). TGF-β1 at 1 ng/ml, increased BSP mRNA levels in ROS 17/2.8 cells ~8-fold; the stimulation was first evident at 3 hr, reached maximal levels at 12 hr and slowly declined thereafter. Since the stability of the BSP mRNA was not significantly affected by TGF-β1, and nuclear "run-on" transcription analyses revealed only a ~2-fold increase in the transcription of the BSP gene, most of the increase in BSP mRNA appeared to involve a nuclear post-transcriptional mechanism. Moreover, the effects of TGF-β1 were indirect, since the increase in BSP mRNA was abrogated by cycloheximide (28 μg/ml). To identify the site of transcriptional regulation by TGF-β1, transient transfection analyses were performed using BSP gene promoter constructs linked to a luciferase reporter gene. Constructs that included nt −801 to −426 of the promoter sequence were found to enhance transcriptional activity ~1.8-fold in cells treated with TGF-β1. Within this sequence, ~500 nt upstream of the transcription start site, a putative TGF-β activation element (TAE) was identified that contained the 5′-portion of the nuclearfactor-1 (NF-1) canonical sequence (TTGGC) overlapping a consensus sequence for activator protein-2 (AP-2). The functionality of the TAE was shown by an increased binding of a nuclear protein from TGF-β1 stimulated cells in gel mobility shift assays and from the attenuation of TGF-β1-induced luciferase activity when cells were co-transfected with a double-stranded TAE oligonucleotide. Competition gel mobility shift analyses revealed that the nuclear protein that binds to the TAE has similar properties to, but is distinct from, NF-1 nuclear protein. These studies have therefore identified a TGF-β activation element (TAE) in the rat BSP gene promoter that mediates the stimulatory effects of TGF-β1 on BSP gene transcription. J. Cell. Biochem. 65:501–512.

Key words: bone sialoprotein; gene regulation; mineralized tissues; TGF-β1; transcription

TGF-β1 is a member of the transforming growth factor-β (TGF-β) superfamily of cytokines that control growth and differentiation in a wide variety of cell types [Massague, 1987]. Although platelets are the most concentrated source of TGF-β1 in the body [Assoian et al., 1983], the high yield of TGF-β1 from bone indicates that the greatest number of TGF-βs are present in skeletal tissues [Hauschka et al., 1986]. TGF-β1 released by platelets in fracture sites, or from the bone matrix by osteoclasts during bone remodeling, can stimulate the formation of bone extracellular matrix, but not

Abbreviations used: AP-1, activator protein-1; AP-2, activator protein-2; BSP, bone sialoprotein; CRE, cyclic AMP response element; LUC, luciferase; NF-1, nuclear factor-1; TAE, TGF-β activation element; TGF-β, transforming growth factor-β; VDRE, vitamin D response element.

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mineralization, which appears to be suppressed by the downregulation of alkaline phosphatase (ALP) activity [Wrana et al., 1988]. However, in combination with the related bone morphorgenetic proteins (BMPs), which stimulate ALP [Takuwa et al., 1991], TGF-β1 may promote the formation of mineralized bone [Sodek et al., 1994].

Bone sialoprotein (BSP) is a sulfated and phosphorylated glycoprotein found almost exclusively in mineralized connective tissues [Oldberg et al., 1988; Fisher et al., 1990; Zhang et al., 1990]. Our previous studies have demonstrated that BSP mRNA expression is essentially restricted to fully differentiated cells of mineralized connective tissues [Chen et al., 1991] and that the expression of BSP is developmentally regulated [Chen et al., 1992]. Further, the temporospatial deposition of BSP into the extracellular matrix [Chen et al., 1991, 1992; Kasugai et al., 1992] and the ability of BSP to nucleate hydroxyapatite crystal formation [Hunter and Goldberg, 1993] indicate a role for this protein in the initial mineralization of bone, dentin, and cementum. Thus, regulation of the BSP gene appears to be important in the differentiation of osteoblasts and for bone matrix mineralization. To study the transcriptional regulation of BSP gene expression, we have cloned and sequenced the rat and human BSP gene promoters [Li and Sodek, 1993; Kim et al., 1994]. This region includes a functional, inverted TATA element (nt −24 to −19) [Li et al., 1995] that overlaps a vitamin D-response element (VDRE) [Kim et al., 1996]. In addition, putative sites of regulation through an inverted CCAAT box (−50 to −46), a cyclic AMP response element (CRE) (−75 to −66) and an AP-2 site (−497 to −490) have been identified in the proximal promoter. Further upstream, a glucocorticoid response element (GRE) overlapping an AP-1 site has been characterized [Ogata et al., 1995a; Yamauchi et al., 1996].

Transcriptional regulation of eukaryotic genes involves complex interactions between *cis*-acting DNA sequences and *trans*-acting protein factors. In the regulation of gene transcription by TGF-β1, both enhancer and suppressor elements have been described. A TGF-β1 activation element (TAE) has been identified in the mouse α2(I) collagen [Rossi et al., 1988] and rat α1(I) collagen gene [Ritzenthaler et al., 1991, 1993]. The nucleotide sequence of these TAEs is very similar, but not identical, to the nuclear factor-1 (NF-1) binding site. A functional TGF-β1 inhibitory element (TIE) has been characterized in the rat transin/stromelysin gene and TGF-β1 inhibition of expression shown to be mediated by the binding of a Fos-containing protein complex to the TIE sequence [Kerr et al., 1990]. In a recent study, an AP-1/CRE element in the proximal promoter of the rat osteocalcin gene has been identified as a target of TGF-β1 supression [Banerjee et al., 1996].

Here we show that TGF-β1 stimulates BSP expression in ROS 17/2.8 cells through a combination of transcriptional and nuclear post-transcriptional regulation and that the increased transcription of the BSP gene is mediated through at TAE located between nt −500 to −484 in the rat BSP promoter.

METHODS

Cell Culture

The rat clonal cell line, ROS 17/2.8 (generously provided by Dr. G.A. Rodan) was used in these studies as an osteoblastic cell line that synthesizes BSP. Cells grown to confluence in 60-mm tissue culture dishes in α-MEM medium containing 10% fetal calf serum (FCS) were changed to α-MEM without serum and incubated with or without 1 ng/ml TGF-β1 (porcine platelet-derived TGF-β1 from R&D Systems, Minneapolis, MN) in the absence or presence of 28 μg/ml cycloheximide for time periods extending over 3-24 hr to determine possible direct and indirect effects of TGF-β1 on the expression of BSP mRNA. To determine the effect of TGF-β1 on the stability of BSP mRNA, cells were first incubated for 12 hr in the presence or absence of 1 ng/ml TGF-β1 and the incubation continued for up to 24 hr in the presence of 60 μM of the transcription inhibitor, 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB). RNA was isolated from triplicate cultures at various time intervals and analyzed for the expression of BSP mRNA by Northern hybridization as described below.

Northern Hybridization

Total RNA from the ROS 17/2.8 was extracted with guanidium thiocyanate and, following purification, 15-μg aliquots of RNA were fractionated on 1.2% agarose gel and transferred onto a Hybond N membrane, as described previously [Overall et al., 1991]. Hybridizations were performed at 42°C with either a

^{32}P-labeled rat BSP or a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. Following hybridization, membranes were washed four times for 5 min each at 21°C in 300 mM sodium chloride, 30 mM trisodium citrate pH 7.0 containing 0.1% sodium dodecyl sulfate (SDS). This was followed by two 20-min washes at 55°C in 15 mM sodium chloride, 1.5 mM trisodium citrate pH 7.0, 0.1% SDS. The hybridized bands, representing the two polyadenylated forms (1.6-kb and 2.0-kb) of rat BSP mRNA, were scanned in a Bio-imaging analyzer (Fuji BAS 2000, Japan).

Nuclear "Run-on" Transcription Analysis

To determine the effects of TGF-β1 on BSP gene transcription, nuclear "run-on" analysis was performed as described previously [Overall et al., 1991]. Briefly, confluent ROS 17/2.8 cells were cultured for 12 hr in the presence or absence of 1 ng/ml TGF-β1 in α-MEM without serum. The cell layers were scraped from the dishes and the nuclei isolated by centrifugation, washed, and then suspended in transcription "run-on" buffer [120 mM Tris–HCl, 50 mM NaCl, 4 mM $MnCl_2$, 0.25 mM EDTA, 350 mM $(NH_4)SO_4$, 34 U/ml RNA guard (Pharmacia, Piscataway, NJ)]. Nucleotides and [^{32}P-UTP] (spec act 3,000 Ci/mmol, NEN, Boston, MA) were added and the reaction mixture incubated for 1 hr at 30°C to initiate transcription. The nuclei were then lysed, the genomic DNA sheared by brief sonication, and RNA collected by centrifugation. Following phenol extraction and isopropanol precipitation, the radiolabeled RNA was used for hybridization with 0.1 μg samples of BSP, osteopontin, ALP, type I collagen cDNA, and control plasmid DNA that had been blotted directly onto a Hybond N membrane. Hybridization and subsequent scanning was performed as described above for the Northern hybridization.

Transient Transfection Assays

Exponentially growing ROS 17/2.8 cells were used for the transfection assays. At 24 hr after plating, cells at 50–70% confluence were transfected using the DEAE–dextran method [Ausubel et al., 1989]. The transfection mixture included 1 μg of a luciferase (LUC) construct [Ogata et al., 1995a] and 2 μg pSV-β-galactosidase control vector (Promega) as an internal control. At 15 min after the direct addition of the DNA–DEAE dextran complex to the cell culture, the cells were the placed in α-MEM media containing 10% FCS. After 3 hr, the cells were shocked by treatment with dimethylsulfoxide (DMSO) for 2 min [10% DMSO in phosphate-buffered saline (PBS)] and refed in α-MEM containing 10% FCS. At 1 day post-transfection, cells were deprived of serum for 24 hr; 1 ng/ml TGF-β1 was added for 12 hr prior to harvesting. The luciferase assay was performed according to the supplier's protocol (picaGene, Toyo Inki, Japan), using a Luminescence reader BLR20 (Aloka, Japan) to measure the luciferase activity. In some experiments, TAE double-stranded oligonucleotides (2 μg), corresponding to the BSP promoter sequence [Ogata et al., 1995a] beginning at −505 (5′-AAAGCCTTGGCAGCCCGGCTGGCT), were co-transfected with the luciferase plasmids to determine whether they could compete for factors stimulating promoter activity in the presence of TGF-β1.

Gel Mobility Shift Assays

Confluent ROS 17/2.8 cells in four T 75 flasks incubated for either 5 or 10 hr with 1 ng/ml TGF-β1 in α-MEM without serum were used to prepare nuclear extracts. Nuclear protein was extracted by the method of Dignam et al. [1983], with the addition of extra proteinase inhibitors; the extraction buffer was 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 25% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 μg/ml aprotinin, pH 7.9. Protein concentration was determined by Bradford's method [Bradford, 1976].

Double-stranded oligonucleotides corresponding to the TGF-β activation element (TAE) in the BSP gene promoter (5′-AAAGCCTTGGCAGCCCGGCTGGCT), and consensus NF-1 (5′-CCTTTGGCATGCTGCCAATAT) [Chodosh et al., 1988] and AP-2 (5′-GATCGAACTGACCGCCCGCGGCCCGT) [Williams et al., 1988] sequences (Promega, Madison, WI) were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Nuclear protein extracts (3 μg) were incubated for 20 min at room temperature (21°C) with 0.1 pM radiolabeled double-stranded oligonucleotide in buffer containing 60 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 mM Tris–HCl (pH 7.5), 0.5 mM DTT, 5% glycerol, and 0.1 μg/ml poly(dI-dC). In some assays, a 20-fold molar excess of nonradiolabeled double-stranded oligonucleotides was included

as competitor. Incubation mixtures were resolved by electrophoresis on 4% nondenaturing acrylamide gels (38:2 acrylamide–bisacrylamide) run at 150 V at room temperature. Following electrophoresis, the gel was dried, and autoradiograms were analyzed by an imaging analyzer. The following double-stranded oligonucleotides were also used as probes or competitors in gel mobility shift assays. An NF-1-like sequence in pLUC3 (5′-TGATTGGCTGCTGAGAGGAG) and an NF-1-like sequence in pLUC7 (5′-CATTTTTTAAATGCCAAAAT) [Ogata et al., 1995a], an AP-1 consensus sequence (5′-CGCTTGATGAGTCAGCCGGAA) [Lee et al., 1987], and a CRE consensus sequence (5′-AGAGATTGCCTGACGTCAGAGAGCTAG) [Roesler et al., 1988] (Promega, Madison, WI).

Statistical Analysis

Triplicate samples were analyzed for each experiment that was replicated to ensure consistency of the responses to TGF-β1. Significant differences between control and TGF-β1 treatment were determined using Student's t-test.

RESULTS

Stimulation of BSP mRNA Expression in ROS 17/2.8 Cells

To study the regulation of BSP expression by TGF-β1, osteoblastic ROS 17/2.8 cells, which have been shown to have osteoblastic characteristics [Majeska et al., 1980] and to express BSP mRNA constitutively [Ogata et al., 1995a], were used. Results of Northern hybridization showed that the treatment with 1 ng/ml TGF-β1 strongly stimulated BSP expression. This stimulation was first evident 3 hr after the addition of TGF-β1 and reached maximal levels ~8 (8.70 ± 2.22)-fold higher than controls, after 12 hr. Subsequently, the mRNA level declined and after 24 hr was ~2 (2.07 ± 0.21)-fold higher than the controls (Fig. 1). Cycloheximide (28 μg/ml) blocked the TGF-β1-induced increase in BSP mRNA expression (Fig. 2) and, as observed previously [Ogata et al., 1995a], BSP mRNA levels declined below the constitutive level of controls not treated with cycloheximide, indicating that protein synthesis is required for both the BSP-mediated stimulation of BSP expression and for maintaining the constitutive expression of BSP. In comparison, GAPDH mRNA was not increased by TGF-β1, nor was it decreased by cycloheximide over the 24-hr period studied.

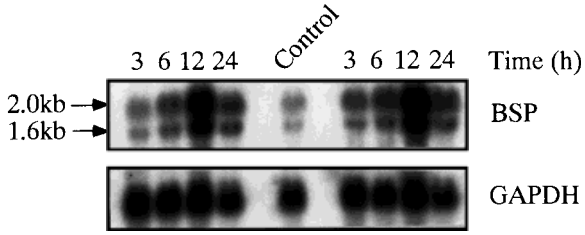


Fig. 1. Northern hybridization analysis of TGF-β1 effects on BSP mRNA expression. A 24-hr time course demonstrating the increase in BSP mRNA following the administration of 1 ng/ml TGF-β1 to osteoblastic cell line ROS 17/2.8. Total RNA was isolated from triplicate cultures harvested after incubation times of 3, 6, 12, and 24 hr and used for Northern hybridization analysis, using [^{32}P]-labeled rat BSP and glyceraldehyde-6-phosphate (GAPDH) cDNA probes. Results of a representative hybridization analysis for control (C) and two series of TGF-β1-treated cells are shown. Differences between TGF-β1-treated cells and controls at the different time points were determined by image analyses.

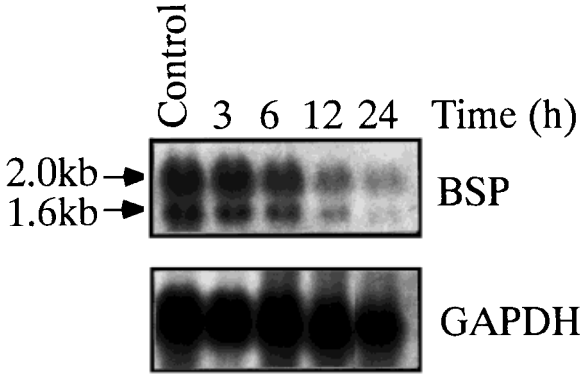


Fig. 2. Effect of cycloheximide on TGF-β1-stimulated BSP mRNA. ROS 17/2.8 cells were incubated with TGF-β1 (1 ng/ml) in the presence of 28 μg/ml of cycloheximide. Control cells were not treated with TGF-β1 or cycloheximide. Total RNA was isolated from triplicate cultures harvested after incubation times of 3, 6, 12, and 24 hr and used for Northern hybridization analysis, using [^{32}P]-labeled rat BSP and GAPDH cDNA probes. A representative hybridization of one of the series of analyses is shown.

Transcription Analysis by Nuclear "Run-on" Assays

To determine whether TGF-β1 increased BSP mRNA levels by activating gene transcription, we performed nuclear run-on assays on nuclei isolated from untreated cultures or cultures treated with TGF-β1 (1 ng/ml) for 12 hr (Fig. 3). The addition of TGF-β1 caused a ~2 (2.03 ± 0.12)-fold increase in BSP gene transcription, as determined by densitometric analysis. Notably, transcription of osteopontin (2.4-fold), ALP

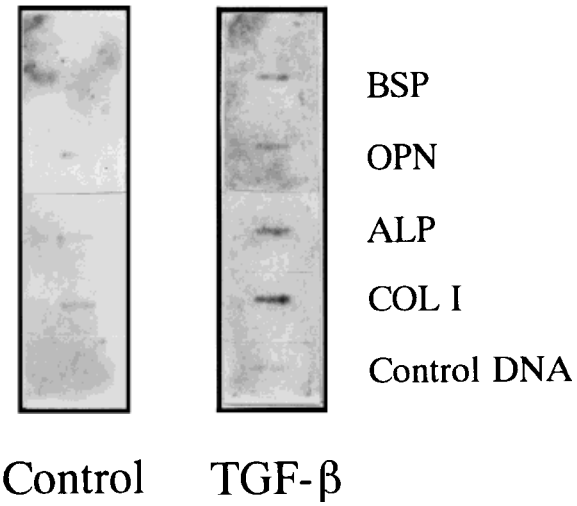


Fig. 3. Nuclear "run-on" transcription analysis. To determine the effects of TGF-β1 on BSP gene transcription, confluent ROS 17/2.8 cells were incubated for 12 hr with (TGF-β) or without (control) 1 ng/ml TGF-β1 and the nuclei isolated and incubated with [^{32}P]-labeled UTP for 1 hr at 30°C to initiate transcription. The radiolabeled RNA produced from triplicate cell cultures was used for hybridization of 100 ng samples of BSP, osteopontin (OPN), alkaline phosphatase (ALP), α1 type I collagen (COLI) and a control plasmid DNA, blotted onto Hybond N membrane. Hybridization and subsequent quantitation by image analysis was performed as described for Northern hybridization. One of the three replicate analyses is shown.

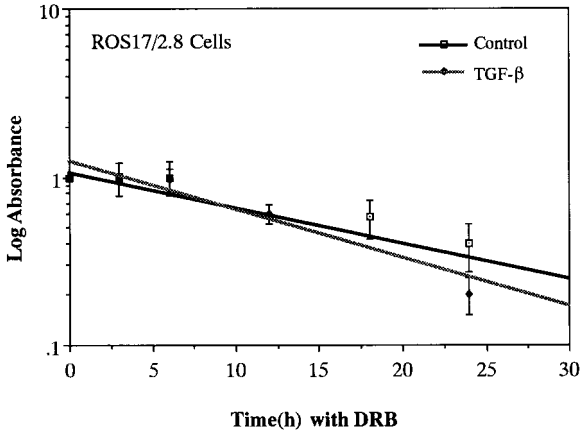


Fig. 4. Analysis of BSP mRNA stability. Confluent cultures of ROS 17/2.8 cells were incubated for 12 hr with or without 1 ng/ml of TGF-β1, prior to the addition of the transcription inhibitor (60μM 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole). Triplicate cultures for each condition were used to prepare total RNA at the times indicated over a 24-hr period. The level of BSP mRNA at each time point was analyzed by Northern hybridization and a half-life of ~16 hr calculated for the mRNA in the ROS 17/2.8 cells.

(1.9-fold), and α1 type I collagen (3.4-fold) were also increased by TGF-β1 stimulation.

Analysis of BSP mRNA Stability

To determine whether the difference in the increased transcriptional activity (~2-fold) and the increase in BSP mRNA (~8-fold) could be explained by an increased stability of the BSP mRNA in response to TGF-β1 treatment, ROS 17/2.8 cells were incubated in the presence of the transcription inhibitor 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole and the BSP mRNA levels determined over a 24-hr period (Fig. 4). From regression analysis, a half-life (t½) of ~16 hr was determined for BSP mRNA in the ROS 17/2.8 cells in the presence and absence of TGF-β1. Thus, the increase in mRNA resulting from TGF-β1 treatment, above that observed by transcription analysis, appears to involve a nuclear post-transcription regulatory mechanism.

Transient Transfection Analysis of Rat BSP Promoter Constructs

Sequences from the 5′-flanking region of the BSP gene were tested for transcriptional activity and responsiveness to TGF-β1 by transient transfection of chimeric constructs in ROS 17/2.8 cells. The constructs used (pLUC3–pLUC10) and their responsiveness to TGF-β1 are shown in Figure 5. pLUC5 encompassing nucleotides −801 to +60 gave a modest, but reproducible, ~1.8 (1.83 ± 0.05)-fold increase in transcription. Varying levels of increase in transcription were also observed in several constructs extending farther upstream. However, deletion of the sequence between -801 and -426 abolished the TGF-β1-mediated stimulation (pLUC3 and pLUC4). Within the DNA sequence that is unique to pLUC5 (between nt −801 and −426), a possible TGF-β activation element (TAE) was identified that contained the 5′-portion of the NF-1 canonical sequence (TTGGC) overlapping a putative AP-2 site (Fig. 6). To determine whether this TAE sequence mediated the TGF-β1 effects on BSP transcription, a double-stranded TAE oligonucleotide was co-transfected with pLUC5 plasmid. In the presence of the TAE oligonucleotide, the increased luciferase activity of pLUC5 caused by TGF-β1 was attenuated (Fig. 7); also, TAE sequence was found to inhibit the TGF-β1 stimulation of pLUC6 and pLUC10 constructs (data not shown), indicating that the oligonuclotide competed for a nuclear factor that bound to the TAE in pLUC5.

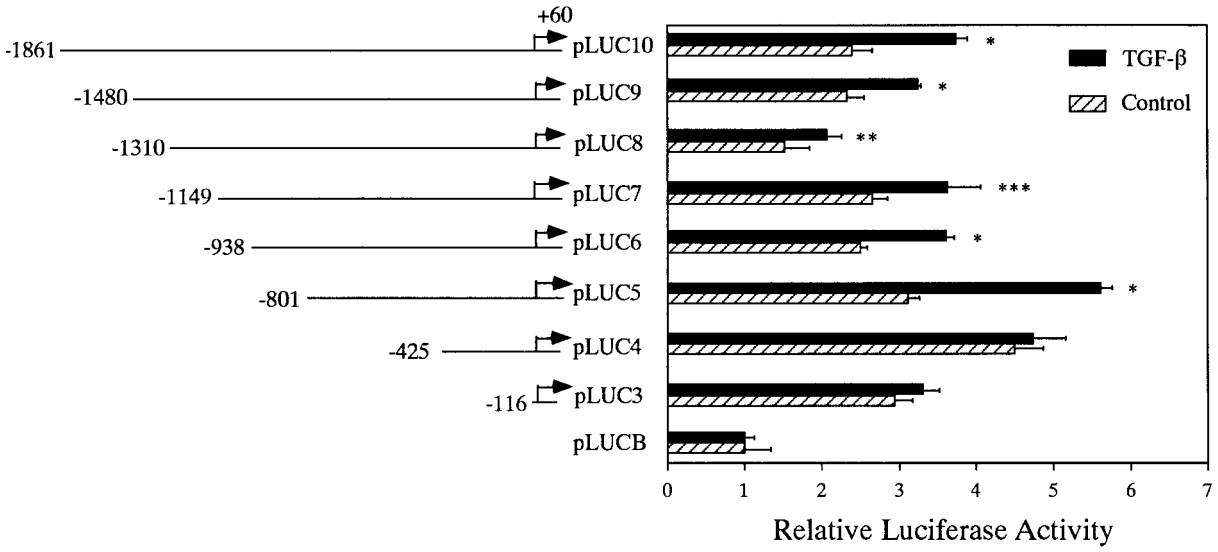


Fig. 5. Transient transfection analysis of rat BSP promoter constructs in the presence or absence of TGF-β1. Transcription activity of chimeric constructs that included various regions of the BSP promoter ligated to a luciferase reporter gene was analyzed following transient transfection of ROS 17/2.8 cells. The results obtained from three separate transfections with constructs. pLUC basic (B) and pLUC3 to pLUC10 have been combined and the values expressed with standard errors. Significant differences from control. $^{*}(P < 0.05)$; $^{**}(P < 0.1)$; $^{***}(P < 0.2)$.

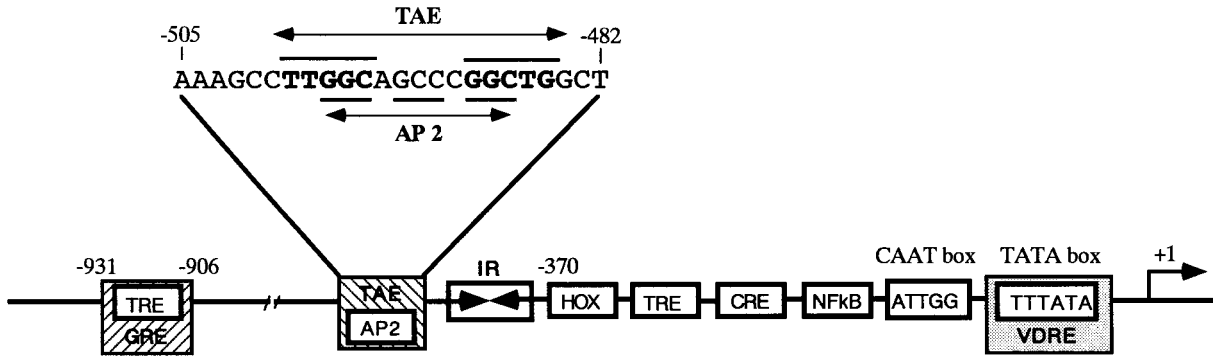


Fig. 6. Site of the TGF-β activation element in the rat BSP promoter. A diagrammatic representation of the BSP gene promoter showing the location of the TAE, which has been expanded, together with the relative positions of the inverted TATA and CCAAT boxes and a number of putative enhancer elements: VDRE that overlaps the inverted TATA box, NFkB, cAMP (CRE), *jun/fos* (TRE), ftz/en (HOX), and an inverted repeat (IR)) that are present within a highly conserved region (nt +1 to −370) of the promoter. A glucocorticoid response element (GRE) overlapping a TRE is also shown located farther upstream.

Gel Mobility Shift Assay

To determine the binding of the TAE sequence to nuclear proteins, the TAE oligonucleotide was end-labeled and incubated with equal amounts (3 µg) of nuclear proteins extracted from proliferating ROS 17/2.8 cells (Fig. 8A, lane 2) and confluent ROS 17/2.8 cells that were either not treated (control, Fig. 8A, lane 3) or treated with TGF-β1 (1 ng/ml) for 5 hr (Fig. 8A, lane 4), or for 10 hr (Fig. 8A, lane 5). There was no significant binding with the probe alone or when incubated with the nuclear extract from proliferating ROS 17/2.8 cells (Fig. 8A, lanes 1 and 2). With nuclear extracts from confluent, control cultures of ROS 17/2.8 cells, a shift pattern was evident (Fig. 8A, lane 3) that closely resembled the AP-2 consensus binding pattern (Fig. 8C, lanes 1 and 2). After stimulation by TGF-β1 for 5 or 10 hr, a strong, but diffuse protein–DNA complex was formed (Fig. 8A, lanes 4 and 5). This pattern of protein–DNA complex induced by TGF-β1 was blocked with cycloheximide (28 µg/ml) for 5 hr (data not shown). Attempts to obtain clearer bands for this complex on 6% acrylamide gels were unsuccessful. Formation of this complex was inhib-

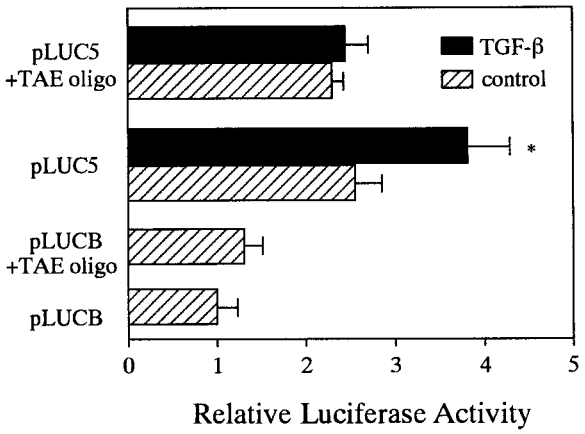


Fig. 7. Attenuation of the TGF-β1 stimulation of pLUC5 transcriptional activity by the TAE oligonucleotide. ROS 17/2.8 cells were transfected with pLUC5 or with pLUC5 + TAE oligonucleotide (2 μg) in the presence or absence of TGF-β1 (1 ng/ml). The results from three separate transfections, have been combined and the values expressed with standard errors. Significant differences from control *($P < 0.05$).

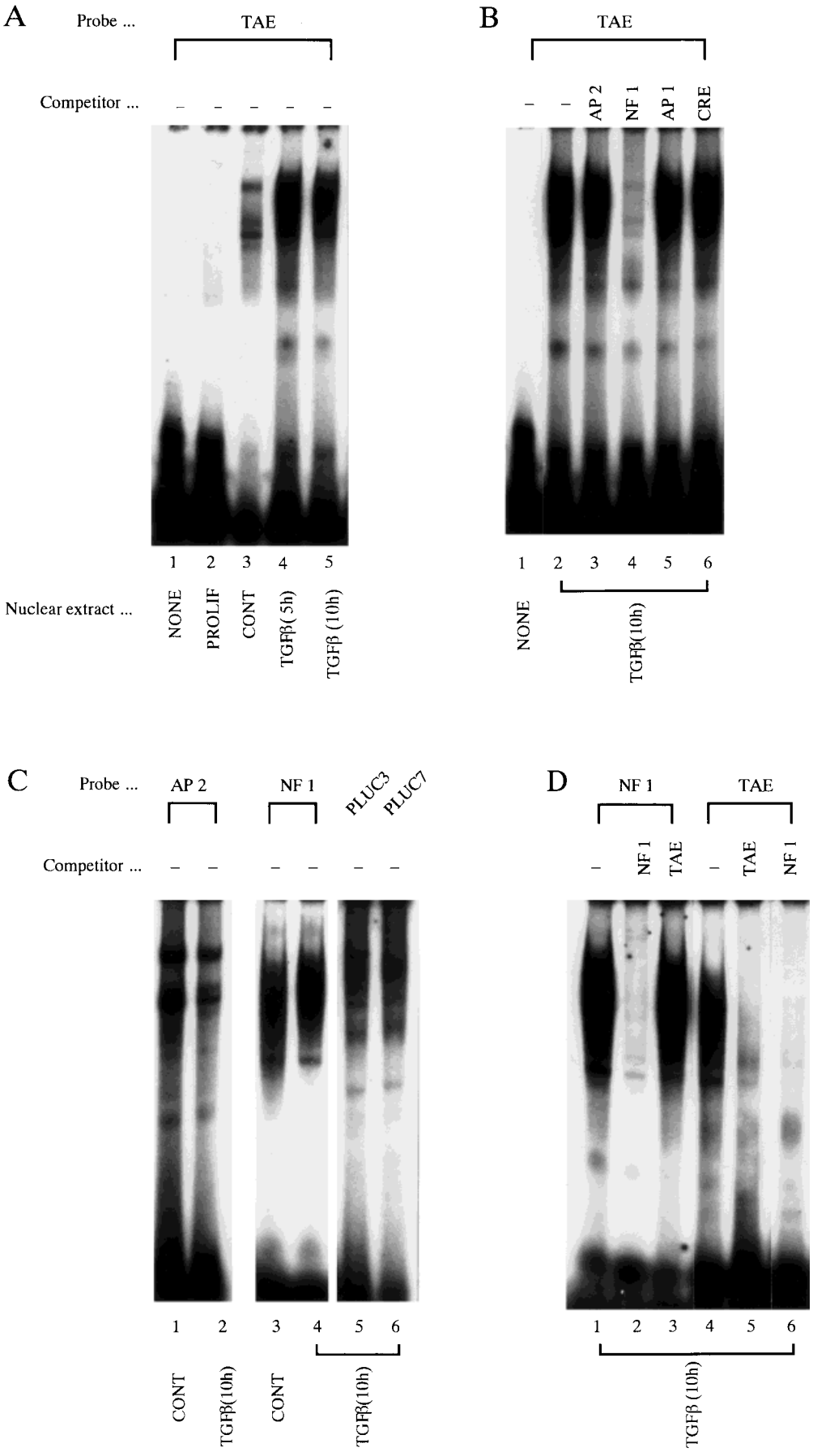
ited by a 20-fold molar excess of the TAE oligonucleotide or the NF-1 oligonucleotide (Fig. 8D, lanes 5 and 6) but was unaffected by a 20-fold excess of AP-2 oligonucleotide (Fig. 8B, lane 3), indicating that the nuclear protein bound to the TAE recognized the NF-1, but not to the AP-2 consensus sequence. Additionally, neither AP-1 nor CREB consensus oligonucleotides at a 20-fold molar excess competed with the TAE-nuclear protein complex (Fig. 8B, lanes 5 and 6), further confirming the specificity of the nuclear protein binding to the TAE sequence. When radiolabelled consensus NF-1 oligonucleotide was incubated with nuclear extracts from TGF-β1-stimulated cells (Fig. 8D, lane 1), a DNA-protein complex, with similar migratory characteristics to the TAE complex (Fig. 8D, lane 4), was generated. However, the NF-1-like sequences in pLUC3 and pLUC7 showed a different binding from the TAE-protein complex (Fig. 8C, lanes 5 and 6). Notably, when the AP-2 or NF-1 were used as the radiolabeled probe, AP-2-protein complex formation was decreased by TGF-β1 stimulation (Fig. 8C, lanes 1 and 2), whereas NF-1 binding was not affected (Fig. 8C, lanes 3 and 4).

To examine whether NF-1 proteins bind to TAE, radiolabeled NF-1 (Fig.8D, lanes 1–3) or TAE (Fig.8D, lanes 4–6) oligonucleotides were incubated with 3 μg of nuclear extract with or without competitors TAE and NF-1 at a 20-fold molar excess. The TAE oligonucleotide did not compete for the protein bound to the NF-1 sequence (Fig. 8D, lane 3). However, the NF-1 competed for the protein in the protein-TAE complex (Fig. 8D, lane 6), indicating that the TAE-binding protein that is upregulated by TGF-β1 is similar to, but distinct from, NF-1 binding proteins.

DISCUSSION

The TGF-β superfamily includes activins and inhibins, bone morphogenetic proteins (BMPs), müllerian inhibiting substance (MIS), as well as the TGF-βs, that regulate the growth and development of various tissues and organs [Sporn and Roberts, 1992]. TGF-β1 displays both mitogenic and chemotactic activity and is characterized by its ability to promote a fibrotic response in connective tissues and to modulate immune system responses [Sporn and Roberts, 1992; Miyazono et al., 1994]. TGF-β1 is relatively abundant in bone tissue [Seyedin et al., 1987]; its synthesis by bone cells appears to be hormonally regulated [Komm et al., 1988; Eriksen et al., 1988; Petkovitch et al., 1987], as well as being autostimulated [Liu et al., 1996]. In accordance with its proposed function in maintaining tissue mass during bone remodeling processes, TGF-β1 stimulates mRNA levels of type I collagen [Ignotz et al., 1987] and osteopontin [Wrana et al., 1991a]. However, the effect of TGF-β1 on gene transcription of bone matrix proteins is clearly selective, since it does not affect the mRNA levels for SPARC (secreted acidic protein rich in cysteine) [Wrana et al., 1991a], while it suppresses expression of both ALP [Wrana et al., 1991a] and osteocalcin mRNA [Noda, 1989].

Our results show that TGF-β1 treatment of ROS 17/2.8 cells increases the steady-state level of BSP mRNA ~8-fold, with increases apparent within 3 hr of TGF-β1 administration (Fig. 1). However, the increase in BSP mRNA appears to be largely due to affects on nuclear post-transcriptional regulation, since transcription of the BSP gene, as measured from nuclear "run-on" analyses, was only increased ~2-fold (Fig. 3), and there was no apparent change in BSP mRNA stability that revealed a half-life of ~16 hr in both the presence and absence of TGF-β1 (Fig. 4). Increases in mRNA through post-transcriptional regulation are not uncommon and have been observed in the autostimulation of TGF-β1 [Liu et al., 1996], and in the stimulation of gelatinase [Overall et al., 1991], and of SPARC by TGF-β in human fibroblasts [Wrana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PLUC7
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– – – – – –
1 2 3 4 5 6
CONT
TGFβ(10h)
CONT
TGFβ(10h)
D
NF 1
TAE
– NF 1 TAE – TAE NF 1
1 2 3 4 5 6
TGFβ (10h)

Figure 8.

TABLE I. Comparison of the Promoter Sequences from TGF-β1-Activated or -Inhibited Genes*

Promoter	Position	Sequence	Reference
BSP	−500	C**TTGGC**AGCCC**GGC**TGG	This study
α2(I) collagen	−308	C**TTGGC**AAGGGCGAG**A**G	Rossi et al., 1988
α1(I) collagen	−1643	GC**TTG**CCCACG**GCCAA**G	Ritzenthaler et al., 1991
Plasminogen activator inhibitor	−562	GC**TGGC**TGCAT**GCC**CTG	Riccio et al., 1992
Transin	−709	GAG**TTGG**TGA	Kerr et al., 1990
NF-1 consensus		T**TTGGC**ATGCT**GCCAA**T	Chodosh et al., 1988
AP-2 consensus		TGACCGCCCGCGGCCCGT	Williams et al., 1988

*The promoter regions of a number of genes demonstrated to be activated (BSP, α2(I)collagen, α1(I)collagen, and plasminogen activator inhibitor) or inhibited (transin) by TGF-β1 were compared with NF-1 and AP-2 consensus sequences. NF-1 sequences are indicated in bold. Underlines designated the AP-2 sequences.

et al., 1991b], as well as in the glucocorticoid-stimulated increase in BSP mRNA [Ogata et al., 1995a]. Indeed, the glucocorticoid-stimulated increases in BSP mRNA expressed in ROS 17/2.8 cells show a similar pattern of regulation, as observed in this study; a modest increase in transcription mediated through a GRE–AP1 complex accompanied by a larger stimulation via an apparent post-transcriptional regulation [Ogata et al., 1995a]. That the effects of TGF-β1 on BSP mRNA were mediated by another protein was indicated initially from the effects of cycloheximide, which blocked most of the increase in BSP mRNA (Fig. 2) and inhibited appearance of the protein-TAE complex seen in the gel mobility shift assay induced by TGF-β1. As demonstrated by gel mobility shift analysis, the nuclear extracts from non-mineralizing cells that do not express the BSP gene, such as human gingival fibroblasts and human periodontal ligament cells [Ogata et al., 1995b], did not bind to TAE sequence after TGF-β1 stimulation for 5 h, suggesting that the factor in ROS 17/2.8 nuclear extracts could be bone cell specific (Y. Ogata and H. Sugiya, unpublished results).

The results of luciferase analyses (Fig. 5), using 5′-deletion constructs of the rat BSP promoter sequence, identified a potential TGF-β-responsive element (TAE) about −500 bp upstream of the transcription start site. Within this region of the promoter, a sequence with the characteristics of a TAE was identified at nt −500 to −484. Further, the functionality of this regulatory element was shown in co-transfection assays using a synthetic oligonucleotide encompassing this site to abrogate the TGF-β1 effects on BSP transcription (Fig. 7). Notably, the TAE sequence in the rat BSP promoter is very similar to TGF-β response elements found in the mouse α2(I) collagen promoter between nt −308 and −292 [Rossi et al., 1988], in the rat α1(I) collagen promoter between nt −1643 and −1627 [Ritzenthaler et al., 1991, 1993] and in the human plasminogen activator inhibitor promoter between nt −562 and −546 [Riccio et al., 1992] (Table I). There are also some similarities to the TGF-β1 inhibition element (TIE) in the rat transin–stromelysin promoter between nt −709 and −700 [Kerr et al., 1990] (Table I). The TGF-β1 inhibition element (TIE) specially binds a nuclear protein complex from TGF-β1-stimulated rat fibroblasts, and this complex contained the c-*fos* proto-oncogene product [Kerr et al., 1990]. In the study conducted by Banerjee

Fig. 8. Identification of a TAE-binding nuclear protein using gel mobility shift assays. **A:** Radiolabeled double-stranded TAE were incubated for 20 min at 21°C with nuclear protein extract (3 μg) obtained from cells incubated without *(lanes 1–3)* or with TGF-β1 at 1 ng/ml for 5 hr *(lane 4)* or 10 hr *(lane 5)*. DNA–protein complexes were separated on 4% polyacrylamide gel in low-ionic-strength Tris–borate buffer, dried under vacuum and exposed to imaging plate for quantitation using an imaging analyzer. NONE, no nuclear extract added; PROLIF, nuclear extract from proliferating cells; CONT, nuclear extract from control confluent cells. **B:** Radiolabeled double-stranded TAE were incubated for 15 min at 21°C with or without a 20-fold molar excess of nonradiolabeled double-stranded oligonucleotide competitors (AP-2, *lane 3*; NF-1, *lane 4*; AP-1, *lane 5*; CRE, *lane 6*) as described in Materials and Methods. Nuclear protein extract (3 μg) was added and the reaction mixture incubated for 20 min at 21°C. DNA–protein complexes were separated on 4% polyacrylamide gel. **C:** Radiolabeled double-stranded AP-2, NF-1, and NF-1-like sequences in pLUC3 and pLUC7 were incubated with nuclear protein extract (3 μg) obtained from cells incubated without *(lanes 1, 3)* or with TGF-β1 at 1 ng/ml for 10 hr *(lanes 2, 4–6)*. **D:** Radiolabeled double-stranded NF-1 and TAE oligonucleotides were incubated with or without 20-fold molar excess of nonradiolabeled NF-1 or TAE for 15 min at 21°C. Nuclear protein extract (3 μg) was added, and reaction mixture was incubated an additional 20 min at the same temperature. DNA–protein complexes were separated on 4% polyacrylamide gel in low-ionic-strength Tris–borate buffer.

et al. [1996], an AP-1/CRE element in the rat osteocalcin gene has been identified as a target of TGF-β1 suppression. This site binds Fra-2, a Fos-related transcription factor, which activates osteocalcin gene transcription. The TGF-β1 appears to block this activation by stimulating the hyperphosphorylation of the Fra-2 through a mechanism that involves signaling through protein kinase C. This mechanism is clearly different from BSP gene activation in this study.

In the original studies of the mouse α2(I) collagen promoter, Rossi et al. [1988] concluded that an NF-1 binding site was the target of transcriptional activation by TGF-β1, whereas closely associated sites for NF-1 and a USF basic helix-loop-helix transactivator have been implicated as targets for TGF-β1 regulation in the plasminogen activator inhibitor promoter [Riccio et al., 1992]. NF-1 is a cellular protein that binds with high affinity to the adenovirus origin of replication and is required for the initiation of replication of cellular DNA [McQuillan et al., 1991]. Several nuclear proteins can recognize different subsets of transcriptional elements containing the NF-1 sequence [Chodosh et al., 1988]. For example, histone H1 binds to the NF-1 site in the mouse α2(I) collagen gene promoter [Ristiniemi and Oikarinen, 1989]. While there are several NF-1-like binding sequences within the rat BSP promoter, they do not appear to be responsive to TGF-β1.

Ritzenthaler et al. [1993] reported that TGF-β activates rat α1(I) collagen transcription through a TAE site that has similarities to an NF-1 site but recognizes a nuclear protein that is similar to, but distinct from, NF-1 protein. Similar to the rat α1(I) collagen promoter, the TAE region in rat BSP promoter comprises an NF-1-like sequence that overlaps a putative AP-2 element. Notably, in the human growth hormone promoter, an AP-2 binding site overlaps the NF-1 site [Courtois et al., 1990]. Moreover, our DNA binding and competition studies have identified a nuclear protein with properties that are consistent with the NF-1-like protein characterized by Ritzenthaler et al. [1993]. Thus, binding of the nuclear protein to the TAE in confluent ROS 17/2.8 cells was increased following TGF-β1 stimulation (Fig. 8A) and formation of the DNA–protein complex was inhibited by an NF-1 consensus oligonucleotide (Fig. 8B, lane 4) but was unaffected by a AP-2 (Fig. 8B, Lane 3), suggesting that the protein(s) binding to the BSP TAE sequence is likely to be a member of the NF-1 family of nuclear proteins [McQuillan et al., 1991]. However, the TAE sequence did not compete with NF-1 protein binding to the NF-1 consensus sequence (Fig.8D, lane 3), nor was NF-1 binding to the consensus NF-1 sequence increased after TGF-β1 stimulation (Fig. 8C, lanes 3 and 4). Thus, the TAE-binding protein is clearly different from the NF-1 binding protein, as observed in studies of the TAE in the rat α1(I) collagen promoter [Ritzenthaler et al., 1991, 1993].

That the TAE binding protein was not evident in proliferating ROS 17/2.8 cells may relate to the expression of BSP by fully differentiated osteoblasts. This may also explain the more modest increase in transcription observed in the transient transfection assays, which are performed prior to the cells being fully confluent, compared to the increase demonstrated in the nuclear "run-on" analyses that were conducted on confluent, nondividing cells. Also, the incomplete suppression of the TGF-β1-mediated increase by the TAE oligonucleotide in the transient transfection assays could indicate the involvement of flanking sequences for additional factors as observed in the plasminogen activator inhibitor promoter [Riccio et al., 1992].

In this study, we have shown that a TGF-β activation element (TAE) exists in the rat BSP promoter through which the stimulatory effects of TGF-β1 on BSP gene transcription are mediated. Since BSP is expressed by differentiated osteoblasts, and the TAE-binding protein is not apparent in proliferating cells, it is conceivable that the TAE may contribute to the cell-specific expression of the BSP gene during the formation of the bone extracellular matrix.

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