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Structural Analysis and Characterization of Tissue and Hormonal Responsive Expression of the Avian Bone Sialoprotein (BSP) Gene

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Abstract Bone sialoprotein (BSP) is an extracellular matrix protein that has a highly restricted expression to mineralized skeletal tissues. The chicken bone sialoprotein-encoding gene (*bsp*) was isolated and shown to contain two less exons than similar mammalian genes, with the absence of an untranslated 5′ exon and the fusion of the first two exons that encode the signal peptide and amino terminal end of the mature BSP peptide. Primer extension analysis showed one strong transcriptional start point (tsp) in mRNA prepared from embryonic bone. Comparison of the avian *bsp* promoter sequence to those of other genes expressed in vertebrate skeletal tissues, identified the presence of homeobox protein binding sequence motifs for engrailed (en-1) and Msx 2 (Hox 8.1), and two collagen type II gene silencer elements. Two TATA sequences one at −21 bp and the second at −172 bp to the tsp were identified. For the first TATA element no CCAAT sequence was observed at an appropriate *cis* position however two Sp1 sequences (GGGCGG) were identified at −66 and −85 bp. A CCAAT element was seen in an appropriate cis position in relationship to the second upstream TATA, but transient expression analysis in embryonic chicken calvaria osteoblasts using two separate promoter/reporter constructs (+24 to −1244 bp or −121 to −1244 bp), confirmed that only the proximal TATA and Sp1 elements were functional. The +24 to −1244 bp promoter sequence demonstrated 33.6, 13.2, and 3.2 fold activity above base line respectively, within cells prepared from embryonic chicken calvaria bone, cephalic sterna, a cartilage that undergoes mineralization and caudal sterna, a cartilage that does not mineralize during embryogenesis. Only base line activity was observed within cells prepared from embryonic dermal fibroblasts a non-skeletal tissue, which does not express BSP. These same cells demonstrated comparable steady state mRNA levels, corroborating that this segment of promoter DNA had tissue specific activity. A series of nested deletions from the 5′ end of the −1244 construct demonstrated that a portion of the tissue specific regulation was controlled by the presence of a silencer element(s) between −1244 and −620 bp since deletion of this segment of DNA resulted in a 6 fold increase in the promoter activity in dermal skin fibroblasts. The −1244–+24 nt promoter construct was shown to be stimulated by dexamethasome ~1.5 fold over control, inhibited by $1,25(OH)_2D_3$ ~60% of control and was strongly stimulated ~5.0 fold by parathyroid hormone (PTH) in embryonic calvaria osteoblasts. These data define the proximal promoter of the avian *bsp* gene and identify several potential regulatory elements that have been observed in the promoters of other genes expressed in skeletal tissues. These elements imparted both tissue and hormone specific promoter activity to *bsp* expression within skeletal cells. J. Cell. Biochem. 64:77–93.

Key words: skeletal; gene; promoter; regulation

Bone sialoprotein (BSP) is a major non collagenous protein found in the extracellular matrices of skeletal tissues. It is almost exclusively expressed by cells within the skeletal lineage and is localized to areas of mineralized growth cartilage and osteoid, and is synthesized by hypertrophic chondrocytes and osteoblasts [Bianco et al., 1993; Chen et al., 1993]. BSP is characterized by its very acidic nature (pI < 4.0) containing high contents of glutamic acid, sialic acid containing glycosylated side chains, serine phosphate, and tyrosine sulfate [Ecarot-Charrier et al., 1989; Franzen and Heinegard, 1985; Fisher et al., 1987; Gotoh et al., 1990; Midura et

Abbreviations used: *bsp*, Bone sialoprotein encoding gene; BSP, Bone sialoprotein; CAT, Chloramphenicol actetyltransferase enzyme; PCR, Polymerase chain reaction; PTH, Parathyroid hormone; tsp, Transcriptional start point.

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al., 1990]. In previous studies from this laboratory several avian cDNAs were isolated and the complete sequence of this protein was deduced and compared to that of the mammalian forms of this protein [Yang et al., 1995]. These studies revealed that while there is considerable sequence divergence between the avian and the mammalian forms of this molecule, the major functional domains of the proteins were conserved. These domains included: three acidic poly-glu regions; two tyrosine-rich domains; several casein kinase II phosphorylation sites; a number of Asn linked glycosylation sites; and an RGD cell binding motif. Of interest in the chicken BSP was the identification of two additional RGD motifs one of these which exists in the human form of the protein in a comparable position in the primary structure as a cryptic RGD-resistant cell-attachment site [Mintz et al., 1993].

In vitro BSP has been shown to bind to collagen, calcium, and hydroxyapatite and has been demonstrated to be able to initiate calcification [Chen et al., 1991; Hunter and Goldberg, 1993, 1994]. Both mammalian and avian forms of the BSP promote RGD-mediated cell attachment [Gotoh et al., 1990; Oldberg et al., 1988] and the mammalian forms of the protein have been shown to interact with the $\alpha_v\beta_3$ integrin class of cell surface receptor found on osteoclasts [Oldberg et al., 1988; Flores et al., 1992; Helfrich et al., 1992; Ross et al., 1993]. Despite its restricted expression to only cells within the skeletal lineage and its possible roles in both mineralization and resorption, little is known about its tissue specific regulation or its regulation by the calcitropic hormones PTH or $1,25(OH)_2D_3$.

In the studies reported here, the chicken *bsp* gene was isolated and characterized and its genomic structure is compared with the mammalian genes. Its transcriptional start site was identified and the essential sequences for the transcription of this gene were identified by transient expression analysis. Several *cis*-acting elements in the promoter which could mediate the regulation of this gene were characterized. Assay of the *bsp* promoter activity in primary cell cultures prepared from several embryonic tissues containing cells of different skeletal lineages as well as in a culture containing a non-skeletal cell type was carried out. The effects of the calcitropic hormones $1,25(OH)_2D_3$ and PTH and the steroid hormone dexamethasone on *bsp* promoter activity were also examined.

MATERIALS AND METHODS

Screening, Isolation, and Sequencing of Genomic Clones

A λEMBL3 chicken genomic library generated by partial *Mbo*I digestion was obtained from Clonetech Laboratories (Palo Alto, CA). Approximately 2×10^5 clones were screened, using the chicken *bsp* cDNAs [Yang et al., 1995]. One of thirteen positive clones (λBSP2) was more extensively characterized by restriction enzyme mapping and Southern blot analysis [Sambrook et al., 1992]. A 3 kb *Apa*I-*Sal*I fragment (Fig. 1A) was subcloned into pBluescript plasmid (Stratagene, La Jolla, CA) and subsequently used for sequence analysis. DNA primers were generated for sequence analysis, at approximately 200 bp increments on both strands of the 3.0 kb *Apa*I-*Sal*I fragment of the genomic DNA. Oligodeoxynucleotide primers were synthesized using an Applied Biosystems Model 391 DNA Synthesizer (A.B.I., Foster City, CA). Nucleotide sequence determinations [Sanger et al., 1988] were carried out with Sequenase (US Biochemical, Cleveland, OH). Sequence analysis was carried out using HiBio DNASIS software (Hitachi American, Brisbane, CA). All sequence data were read from overlapping sequences on both DNA strands from a minimum of three separate gel analyses for each primer.

Primer Extension

Two different primers were used for these analyses: an antisense 31mer corresponding to +219 to +189 nt, and a 23mer corresponding to +144 to +122 nt of the chicken *bsp* cDNA [Yang et al., 1995]. Primers were labeled with ^{32}P at the 5′ end using polynucleotide kinase [Sambrook et al., 1992]. Primer extension of the chicken *bsp* mRNA was carried out on poly(A)-selected mRNA [Gerstenfeld et al., 1989] purified from 17-day embryonic chicken tibia bones and embryonic chicken calvaria osteoblasts. After the reverse transcription reaction, products were resolved on a 6% sequencing gel against a sequencing ladder made with the control DNA provided in the Sequenase sequencing kit (US Biochemical, Cleveland, OH).

Isolation and Analysis of RNA

Total RNA was isolated using Tri-Reagent™ (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions.

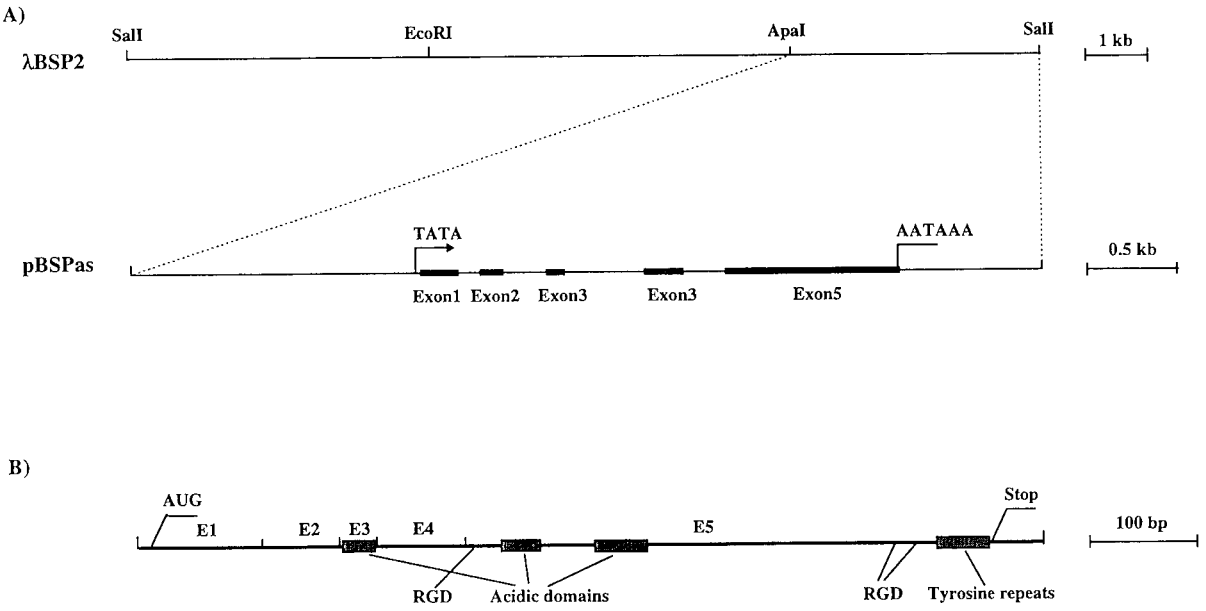

Fig. 1. Restriction map and diagrammatic representation of the exon/intron structure and coding domains of the avian *bsp* gene. The restriction map of λBSP2 (**A**) while a subclone of Ap1-Sal1 fragment in pBluescript is depicted in the middle panel with the exon intron structure denoted by thick and thin lines respectively. **B:** The transcript of the gene with the linear position of the conserved structural domains is denoted.

RNA was resolved on 1% agarose gels containing 2.2 M formaldehyde [Leharach et al., 1977; Thomas et al., 1980] and 5 µg of total RNA based on OD_{260} was loaded per gel lane. Equal loading of the RNA was verified by ethidium bromide staining of the gel before blotting onto Biotrans nylon membranes (ICN Biomedical INC. Aurora, OH). The cDNA (MMPP3) encoding the 3′ sequence of chicken bone sialoprotein [Yang et al., 1995] was used to examine levels of expression for mRNA. ^{32}P-radiolabeled cDNA probes were synthesized [Feinberg and Vogelstein, 1983] and hybridization was carried out at 65°C in 50 mM PIPES, 100 mM NaCl, 50 mM Sodium phosphate pH 7.0, 1 mM EDTA buffer, 5% SDS (w/v), and 60 µg/ml single stranded salmon sperm DNA for 18 h in a rotating hybridization oven (Robins Scientific, Sunnyvale, CA). Autoradiograms were quantified using an LKB Ultra II scanning densitometer (LKB, Broma, Sweden) and values were normalized to 18 S ribosomal RNA obtained by hybridization of each blot to a conserved nucleotide sequence probe of the 18 S ribosomal subunit (Ambion Corp., Austin, TX).

Cell Culture

Osteoblasts were isolated by three sequential trypsin-collagenase treatments of 17-day-old chicken calvariae as previously described [Gerstenfeld et al., 1987]. Only the cells released from the third sequential digest were used for experiments. Cultures were grown for 2 weeks until reaching confluency in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) with media changes every 3 days. At the time that the cultures reached confluency the cultures were switched to BGJb media supplemented with 10% FBS. After 2 days this media was supplemented with 10 mM β-glycerophosphate subsequently after an additional 2 days this media was further supplemented with 12.5 µM ascorbic acid. This media is denoted as "complete media." Chicken embryonic skin fibroblasts were prepared by trypsin/collagenase digestion of 12 day embryonic chicken skin. Cells were plated at a density of 2.5×10^5/100 mm diameter tissue culture dish. These cells were maintained in Dulbeco's modified eagles medium DMEM supplemented with 10% with FBS. Chondrocytes were prepared from the caudal and cephalic halves of 17 day embryonic chicken sterna [Gerstenfeld et al., 1989]. In these experiments the chondrocyte cultures were grown under conditions that were permissive for growth chondrocyte differentiation as previously described for cultures of chondrocytes

prepared from the 12 day embryonic chicken ventral vertebrae columns [Gerstenfeld and Landis, 1991]. In these cultures all experiments were carried out 1 week after the cultures had been placed in "complete media."

Transfection and CAT Assay

Plasmid constructs containing promoter sequences from −1244 to either −121 or +24 nt were subcloned in the sense direction relative to the *cat* gene coding sequence into the pCAT basic vector (Promega, Madison, WI). Nested deletions from the 5′ end of the −1244 to +24 nt of *bsp* promoter were generated by PCR amplification of selected segments of the promoter using primers from −978, −620, −524, and −131 and a down stream primer from +24 nt. Cloning was performed using standard methodology [Sambrook et al., 1992]. All assays were performed in the primary chicken osteoblast and chondrocyte cultures 1 week after switching to complete media. Skin fibroblasts were used one week after plating in culture. DNA was transfected by either electroporation or lipofection. All CAT assays were performed 48 h after transfection. DNA transfections by lipofection were carried out with lipofectamine (Life Sciences Technologies, Gaithersburg, MD). For these studies cells were plated at 5×10^4 cells per 33 mm diameter well in six well dishes. Transfections were then carried as recommended by the manufacturer using a DNA to lipofectamine ratio of 2.5 µg/5 µl of lipofectamine except were otherwise stated. Six hours after transfection the media was adjusted to a final concentration of 20% v/v with FBS. In experiments in which DNA was transfected by electroporation 5×10^6 cells were transfected with 30 µg of the bse/*cat* promoter plasmids using a Bio-Rad Gene Pulser (Bio-Rad, Richmond, CA) as previously described [Rafidi et al., 1994]. After transfection at 24 h, PTH (Bovine 1-34 PTH), Dexamethasone, or $1,25(OH)_2D_3$ were added at a final concentration of 10^{-8} M each. PTH and dexamethasone were from Sigma Chemicals Inc. (St. Louis, MO) and $1,25(OH)_2D_3$ was from Biomol. Inc. (Plymouth Meeting, PA). CAT activity was assayed by liquid scintillation counting [Sleigh, 1986]. Reactions were then counted at 5–10 min increments over a ≥2 h period. The cpms for each sample were plotted against time, and the slope of linear portion of the curve which is representative of the CAT activity was calculated. The final enzyme activities were expressed as cpm of converted ^{14}C-chloramphenicol/min/µg of protein. In order to normalize the CAT activities to account for variations in the transfection efficiency between cell types, the relative amount of plasmid vector in the transfected cells was determined. The total amount of CAT plasmid DNA was determined by extracting the total DNA from one half of each of the samples used to assay the CAT enzyme. Equal proportions of the total DNAs from each sample were then slot blotted in three dilutions and hybridized to the segment of the CAT basic vector excluding the CAT enzyme coding region. Enzyme activities were then normalized to the relative amount of CAT vector in each sample as determined by scanning densitometery of the autoradiograms of the slot blots. CAT assay values were the averages of at least duplicate measurements from three separate transfections of each preparation of cells. All results presented represent the averages of assays from at least three separate preparations of cells.

RESULTS

Sequence and Exon/Intron Structure of the Chicken *bsp* Gene

In order to characterize the *bsp* gene and its regulation it is first necessary to define its sequence and identify possible *cis* acting elements which control its expression. A chicken genomic λEMBL3 library was screened by ^{32}P-labeled chicken *bsp* cDNA [Yang et al., 1995] and 13 positive clones were isolated. The restriction sites of one of the positive clones (λBSP2) was mapped, and an *Apa*I-*Sal*I fragment which hybridized to the cDNA probe in Southern blot experiments was subcloned into pBluescript (Stratagene, La Jolla, CA) (Fig. 1A). The 5′ to 3′ orientation and relative position of the coding sequences within the *Apa*I-*Sal*I DNA fragment were determined by the size of various PCR products, using specific primers localized within the *bsp* coding region and flanking sequences of the cloning vector.

The entire coding region inclusive of introns was sequenced and exons were localized by alignment with the chicken *bsp* cDNA [Yang et al., 1994]. A schematic diagram of the exon/intron structure and the functional domains encoded by each exon is depicted in Figure 1B

while the sequence of the entire coding region is shown in Figure 2. The tsp was determined as described below. The sequence contiguous to the identified translation start site (accATGA) was consistent with the Kozak consensus of translation initiation site [Kozak, 1981, 1986]. The chicken *bsp* gene was found to be organized as follows: a signal peptide fused to a recognition sequence for casein kinase II phosphorylation; a tyrosine rich domain; a poly-Glu domain containing recognition sites for casein kinase II phosphorylation; a domain showing little cross specie similarity; and a large 3′ exon containing a poly-Glu domain several Asn glycosylation linkage sites, a second poly-Glu domain a conserved RGD integrin recognition site and a second tyrosine rich domain. The intron boundary sequences were analyzed and this data is summarized in Table I. As can be seen from this Table these data are consistent with the consensus for known splicing sites [Hernandez et al., 1984; Padget et al., 1986].

Characterization of the Transcription Start Point and Promoter Sequence Analysis

Primer extension analysis was performed to determine the transcription start point. Two different primers were used to provide independent confirmation of the tsp. One strong extension product was identified with both primers, while a minor site could be observed at 24 bp further upstream. These results indicate that transcription starts primarily at a single site. The autoradiograph from one of the two primers in the experiment is depicted in Figure 3. Approximately 1200 bp of the 5′ flanking sequence of the avian *bsp* gene promoter were analyzed and this sequence is presented in Figure 4. Within this figure a number of sequences are denoted that had similarity to known *cis* regulatory elements. Besides the TATA and Sp1 sequences that are identified in the figure, two homeobox protein binding domains to that which are recognized by engrailed-1 (en-1)

TABLE I. Comparison of Exon Intron Junctions in the Human and Avian Bone Sialoprotein Genes*

	EXON	INTRON	EXON
Chicken	missing first non-coding exon		
Human[a]	CTGCCAGAGGgtaagatttaa	.....	ttaattccagAAGCAATCAC
Chicken	CGCCTTCTCG	no intron	GTGAGGAGCT
	A F S		V R S
Human	TGCTTTCTCAgtaagttcttt	.....	tttaaaacagATGAAAAATT
	A F S		M K N
Chicken	GGAGAATGCGgtagggttgtc	.....	ctcctggcagGTGCTGAAGA
	E N A		V L K
Human	AGAAAATGGGgtaattaattt	.....	gtttttgcagGTCTTTAAGT
	E N G		V F K
Chicken	TCGCTACAAGgtgggtctatc	.....	cttcacatagGGCAGTGACT
	R Y K		G S D
Human	TCCAGTTCAGgtaaatataga	.....	atatttttagGGCAGTAGTG
	P V Q		G S S
Chicken	GGAAGGGGGGgtgactatggg	.....	cactccacagGCACCGTCCC
	E G G		A P S
Human	GGAAGAAGAGgtaaggaattt	.....	tttttaacagGAGACTTCAA
	E E E		E T S
Chicken	TGCCCATCAGgtgagctctct	.....	tgtgctgcagGACTGCAAAG
	A H Q		D C K
Human	TCCCAAGAAGgtaacaatgga	.....	ttccttacagGCTGGGGATA
	P K K		A G D

*Coding sequence is denoted in capital letters while lowercase denoted intron sequence. Single letter amino acid codes are denoted under each codon. The consensus for all vertebrate genes is: $A_{62}G_{77}\backslash g_{100}t_{100}a_{60}a_{74}g_{88}$ for the 5′ donor; $y_{87}n_{97}a_{100}g_{100}\backslash G_{55}$ for the 3′ acceptor side. Subscript numbers indicate the percentage of occurrence for that nt in that position. y = Any pyrimidine, n = any nucleotide [Hernandez and Keller, 1983; Padgett et al., 1986].

[a]The deduced exon intron junctions of the avian *bsp* gene were compared to that of the human gene as reported by Kerr et al. [1993].

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Exon 1
AATCAGTGCTGGCCCAGTGGCACCATGAGGAGCGCGCTGCTGCTTGCCTGCCTGCTGGCCACTGCCTCCG   70
                          M  R  S  A  L  L  L  A  C  L  L  A  T  A  S    15

CCTTCTCGGTGAGGAGCTGGCTGCGCAGAGCCCGGGCAGGCGACTCGGAGGAGAATGCGgtagggttgtg  140
A  F  S  V  R  S  W  L  R  R  A  R  A  G  D  S  E  E  N  A              35

ccacgacagcgccggagcattgcagccccccaagctgtggggctgactccctccctctcctggcagGTGC 210
                                                                      V   36
Exon 2
TGAAGAGCCGGCACCGGTACTACCTGTACCGCTACGCCTACCCGCCGCTGCATCGCTACAAGgtgggtct  280
L  K  S  R  H  R  Y  Y  L  Y  R  Y  A  Y  P  P  L  H  R  Y  K           57

atcgggttccgtgggtccccagcctcatgcgcagagatattgccccaaaaagccccgctgggctgaggcc  350
                                                    Exon 3
gtgatgcccatgatactgatgacaatggctctactctcccttcacatagGGCAGTGACTCCTCGGAGGAA  420
                                                      G  S  D  S  S  E  E   64

GAGGGGGACGGCTCAGAGGAGGAGGAGGAAGGGGGGGtgactatgggcctccagcgtgcagcaattcagg  490
 E  G  D  G  S  E  E  E  E  E  G  G                                      76

gacttgcatctgaaccatcctacagctttttggatgagtgtgaaccccttgtcgtgtctcctcctcactc  560
      Exon 4
cacagGCACCGTCCCATGCAGGTACTCAGGCAGCAGGCGAGGGGCTGACCCTCGGGGATGTGGGGCCGGG  630
      A  P  S  H  A  G  T  Q  A  A  G  E  G  L  T  L  G  D  V  G  P  G   98

AGGTGACGCTGCGTCTGCCCATCAGgtgagctctctgcattcagagccctgaggtgtggctgatgccggg  700
  G  D  A  A  S  A  H  Q                                                 106
                          Exon 5
ttatttattgctctgtccctgtgtgctgcagGACTGCAAAGGGGGCCAGAAGGGCACACGGGGTGACTCG  770
                                D  C  K  G  G  Q  K  G  T  R  G  D  S    119

GGCGATGAGGACAGTGATGAGGAAGAGGAGGAAGAGGAGGAGGAGGAGGAAGAAGAGGTAGAGGAGC  840
 G  D  E  D  S  D  E  E  E  E  E  E  E  E  E  E  E  E  E  V  E  E     142

AGGATGTCAGTGTCAATGGGACCAGCACCAACACCACAGCAGAGACACCCCATGGGAACAACACCGTGGC  910
Q  D  V  S  V  N  G  T  S  T  N  T  T  A  H  T  P  H  G  N  N  T  V  A  166

AGCTGAGGAGGAGGAGGATGATGATGAGGAGGAGGAAGAGGAAGAAGAGGAGGAGGAGGAAGCTGAAGCC  980
  A  E  E  E  E  D  D  D  E  E  E  E  E  E  E  E  E  E  E  A  E  A     189

ACCACCGCTGCTGCCACCACTGCGCAGGATGAGGTGACCACGTTGGGCGATGAGCAGCGCTCTGAGGTCA 1050
 T  T  A  A  A  T  T  A  Q  D  E  V  T  T  L  G  D  E  Q  R  S  E  V    212

CTACAGCTGGGGAGCAGTGGGAGTACGAGGTGACAGTGGGAGCCCGCGGGGATGAAGGTCCCACTGAGAG 1120
T  T  A  G  E  Q  W  E  Y  E  V  T  V  G  A  R  G  D  E  G  P  T  E  S  236

CAGCTATGGGGACCAGGAGGAGCCAGCACGTGGGGACAGCTACCGCGCCTATGAGGATGAGTACGGCTAC 1190
  S  Y  G  D  Q  E  E  P  A  R  G  D  S  Y  R  A  Y  E  D  E  Y  G  Y   259

TACAAGGGGCATGGCTATGACATGTATGGGCAGGATTACTACTACAACCAGTGAGAGAGTGGTGGAACGC 1260
 Y  K  G  H  G  Y  D  M  Y  G  Q  D  Y  Y  Y  N  Q                     276

GCAACACCACCCTGGCCTGAGTATTGCCATCCATGTATCGCAGAAACGCAATAAAGCAACAAGTGGAGCT 1330

CGTG                                                                   1334
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Fig. 2. The complete nucleotide sequence of the genomic DNA encoding the chicken BSP gene. Lowercase letters denote intron sequences and upper case letters denote exon sequences. Sequences are numbered continuously from the 5′ *tsp* deduced by primer extension analysis and at the 3′ end terminates in the polyadenylation signal. The translational start site, the two in-frame stop codons and the polyadenylation signal are underlined. Single letter codes denoting the amino acids which are encoded by each exon are indicated in the figure.

(TCAATTAAAG) [Levine and Hoey, 1988] were found at −138 to −129 nt and −966 to −949 nt (CCAATTAAAT). A similar sequence (TCAATTAAAT) is also found in human and rat *bsp*, the rat osteocalcin promoter and the chicken and mouse osteopontin promoters (Table II) [Kerr et al., 1993; Li and Sodek, 1993; Towler et al., 1994; Denhardt and Guo, 1994; Rafidi et al., 1994; Hoffmann et al., 1994]. A second homeobox binding sequence (TCTAATTAC) containing the core recognition sequence TAATT [Catron et al., 1993] which is similar to that recognized by Msx 2 or Hox 8.1 were identified. Two sequences, CACCTCT (−850 to −844 nt) and CACCTGC (−1214 to −1208 nt), that resemble the silencer previously identified in the type II collagen gene (COL II silencer) [Savagner et al., 1990; Ryan et al., 1990] were also identified. In addition, a Vitamin D_3 response element (VDRE) [Demay et al., 1990; Markose et al., 1990], Ap1 [Angel, 1987], and a half-site of a cAMP response element (CRE) [Montminy et al., 1986; Comb et al., 1986] are identified in Figure 4. Table II summarizes a comparison of five of these sequences (the homeobox sequences, the COL II silencer elements and the CRE/AFT binding sequences) specifically found in the chicken *bsp* gene promoter that were identified in other species of the *bsp* gene promoter as highly conserved elements, as well as in other genes specifically expressed by cells within the skeletal lineage.

Within the proximal promoter of the chicken *bsp* gene two TATA boxes were identified by sequence analysis: the distal one is at −171 nt, it is a inverted TATA box, TTATAT, and a CCAAT motif is located 30 bp 5′ to this sequence. The second more proximal TATA is at −26 bp, however a CCAAT motif is not seen appropriately arranged in a *cis* position to this sequence, instead two Sp1 elements located at −66 and −85 bp are observed. The presence of only one start site for the primer extension analysis and the position of the two TATA sequences relative to the identified tsp suggests that only the proximal TATA is functional in the transcription of the gene. To further examine the function of the both TATA sequences, two promoter/CAT constructs (CAT +24 to −1244 and CAT −121 to −1244) were made, CAT +24 to −1244 contains both TATA sequences while the CAT −121 to −1244 lacks the proximal TATA box. This is shown in the diagram in Figure 5c. These constructs were transfected and transiently expressed in primary osteoblast cell populations prepared from 17 day chicken embryos. Figure 5a shows the increase of the product of the CAT enzyme assays over a 2–3 h time period. The linearity of the reactions are indicative that the assay conditions used were indeed at the initial rate of the CAT reactions, thus the slopes of the curves are representative of the CAT activities. Figure 5b shows the mean result of triplicate measurements for three sepa-

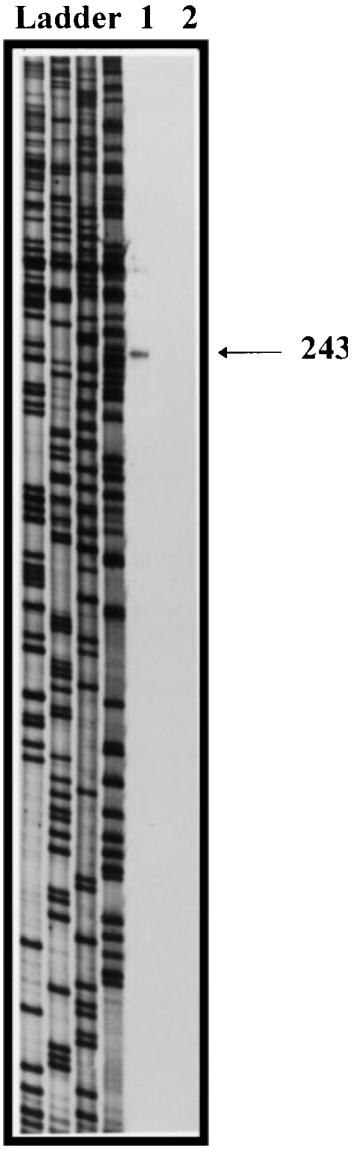

Fig. 3. Primer extension analysis of chicken BSP mRNA. Autoradiogram of a primer extension experiment with RNA purified from the bone of chicken embryo. Primer is located +219 to +189 nt of the reported cDNA sequence [Yang et al., 1995]. The size of the extension product was determined relative to the sequencing ladder made with the control DNA provided in the Sequenase sequencing kit (US Biochemical, Cleveland, OH).

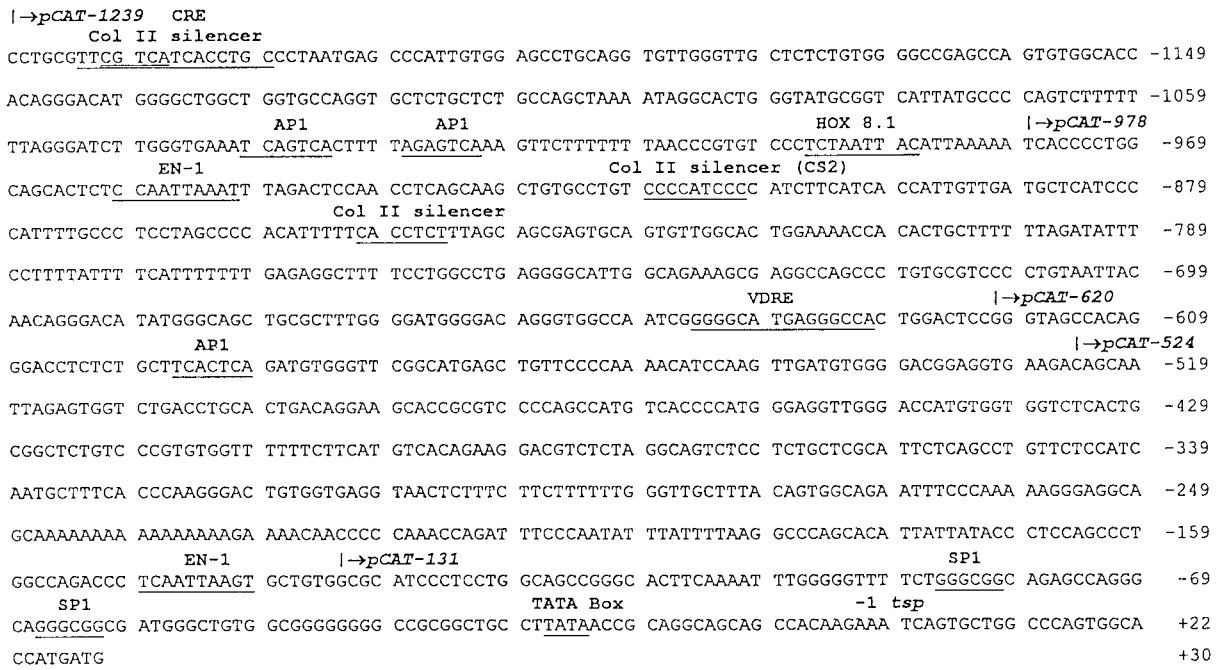

Fig. 4. Nucleotide sequence of the avian bone sialoprotein promoter. ~1.2 kb of 5′ flanking region of chicken *bsp* depicted in the figure. Positions of putative *cis* acting regulatory sequences are underlined. The positions of individual pCAT promoter constructs are denoted above the sequence.

rate experiments, demonstrating that from preparation to preparation of the primary cells, the promoter activity behaves in a reproducible fashion. From this experiment it can be concluded that the distal TATA, even with the upstream CCAAT like element, is not functional and only the proximal TATA is utilized in primary osteoblast. This result is also consistent with the Northern analysis in which only one mRNA specie was seen when hybridized to the *bsp* cDNA probe [Yang et al., 1995]. However, the possibility can not be ruled out that the other TATA may be used at certain stages of avian skeletal development or in response to certain hormonal signals.

Tissue Specific Expression of the *bsp* Gene is Controlled by *cis* Element(s) Within the 1200 bp Promoter Sequence

To test whether the *cis* element(s) which control the tissue specific expression of the *bsp* gene are localized within the first 1200 bp of the proximal 5′ promoter, the expression of *bsp* promoter (+24 to −1244)/CAT construct was examined in three primary cell populations prepared from different embryonic chicken skeletal tissues, which express varying levels of BSP mRNA, and one tissue that does not express BSP (see below). Osteoblasts were prepared from 17 day calvaria and two populations of chondrocytes were prepared from 17 day embryonic sterna: cephalic sterna chondrocytes which undergoes hypertrophic differentiation both in vivo and in vitro and caudal sterna chondrocytes which does not differentiate in culture to a hypertrophic state. Dermal fibroblasts were examined as a representative non-skeletal cell type that does not express this gene.

In order to assess the discrepancy of the transfection efficiency for different cells used in the experiments reported here, the amount of transfected DNA/dish was directly measured and the promoter efficiency was calculated relative to the amount of transfected DNA. Initial control experiments were carried out to demonstrate that the amount of the transfected DNA was proportional to the amount of CAT activity. 0.25–3 μg of the SV40-driven CAT control DNA were transfected into primary chick osteoblasts and the relationship of the CAT activity to amount of transfected DNA were determined (Fig. 6). Half of each cell sample were harvested for assay of the CAT activity and as can be seen, within this range of transfected DNA quantities, a linear relationship between the input

TABLE II. Comparison of Regulatory Sequences Identified in the Avian *bsp* Promoter to Those Functionally Identified in Other Skeletal Specific Genes*

Homeobox-binding elements		EN-1			HOX 8.1				
Consensus		AAACCTT	**CAATTAAAT**	TC		AC	**TAATT**	GG	
BSP (Chick 1)	−966	CACTCTC	**CAATTAAAT**	TT	−949	−111	TC	**TAATT**	AC	−103
BSP (Chick 2)	−154	AGACCCT	**CAATTAAAG**	TG	−148					
BSP (Human)	−208	AAACCTT	**CAATTAAAT**	TC	−191	−377	AC	**TAATG**	TA	−369
BSP (Rat)	−200	CAGCCTT	**CAATTAAAT**	TC	−183	−385	CC	**TAATT**	CA	−376
OC (Rat)	−1262	CAACCTT	**CACTTTAAT**	AT	−1242	−84	AC	**TAATT**	GG	−92
OPN (mouse)	−36	GGACCAG	**CATTTAAAT**	TC	−19	−313	AC	**TAATT**	CA	−306
OPN (chick)	−1050	ACACCTT	**CAAAAGCTG**	CA	−1033	−745	CT	**CAATT**	GA	−737
COL2 (rat)		n.d.				+353	CC	**TAATT**	CT	+361
Silencer elements		COL2 (S1) CR-1				COL2 (S2)				
COL2 (Rat)	−666	ACCCTCTCTT		−657		−435	CCCATCCC		−428	
BSP (Chick)	−849	CACCTCTTTA		−843		−917	CCCATCCC		−910	
BSP (Human cp)	+11	GCCCTCTCAC		+2		+1678	TCCATCCC		+1685	
BSP (Rat)	−408	CACCTCTTGT		−400		−396	ACCATCTT		−389	
OC (Rat1)	+38	ACCCTCTCTC		+63		−669	CCCATCAA		−662	
OC (Rat2)	+95	ACCCTCTCTG		+104						
OPN (Mouse)	−589	ACTCTCTGTT		−580		−337	TTCATCCC		−330	
OPN (Chick)	−542	ATTCTCTCTG		−533		−469	CCCATCAG		−462	
CRE/ATF										
Consensus		TGACG	C/T	C/A	G/A					
BSP (Chick cp)	−1231	TGACG	A	A	C	−1223				
BSP (Rat)	−176	TGACG	T	C	G	−168				
BSP (Human)	−74	TGACA	G	T	G	−56				

*Sequence comparisons were made between 5′ promoter regions of various genes that are expressed in a restricted or at very elevated level in skeletal cells. The five separate sequences identified in this study were based on comparisons with functional analysis that demonstrated that these elements play a role in the regulation of one or more of these genes. COL II silencer elements were as identified in Savagner et al. [1990] and Frenkel et al. [1995]. The element identified as COL2 (S1) has sequence similarity with the middle repetitive CR-1 element identified in both mammalian and avian genomes, and found as a tissue non-specific silencer in numerous genes [Scott et al., 1987; Baniahmad et al., 1987]. The identification of Hox 8.1 was based on the studies of Towler et al. [1995] and Ducy and Karsenty [1995]. The identification of en-1 engrailed sequence was solely based on sequence similarity [Levine and Hoey, 1988]. Core elements of en-1 and Hox 8.1 are denoted by bold letters [Catron et al., 1993 and Levine and Hoey, 1988]. The identification of the CRE/ATF consensus sequence is as described in Faisst and Meyer [1992] and the identification of the CRE in rat BSP promoter is as reported by Li and Sodek [1993]. n.d. = Not detected. cp = Complementary strand.

DNA and enzyme activity was obtained (panel A). Total DNA was extracted from the other half of each sample, and 2 µg of this DNA was digested with *HindIII* and *BamHI* to release the plasmid backbone of the vector. Analysis of this sample by Southern hybridization demonstrated that only the plasmid backbone and not the CAT coding sequence or its transcripts was being examined. Panel B of this figure depicts that the actual amount of plasmid DNA within the total extracted DNA from each sample does increase in a linear fashion in relationship to the input DNA. Panel C shows the ratio of measured amount of CAT activity to the measured amount of transfected DNA. The near 1:1 proportionality between the amount of transfected DNA within the cell and CAT activity demonstrates that there is a high degree of correlation between actual amount of transfected DNA and the CAT activity in the range tested.

The transcriptional specificity of the *bsp* promoter was then tested in the four different embryonic cell types (Fig. 7A–C). The CAT activities were assayed and the values normalized to the amount of transfected plasmid DNA found in each sample (Panel A). As can be seen from this analysis variation in the *bsp* promoter's activity in these different cell types were observed, with the osteoblasts showing the greatest activity with progressively lesser activities seen in the cephalic, and caudal chondro-

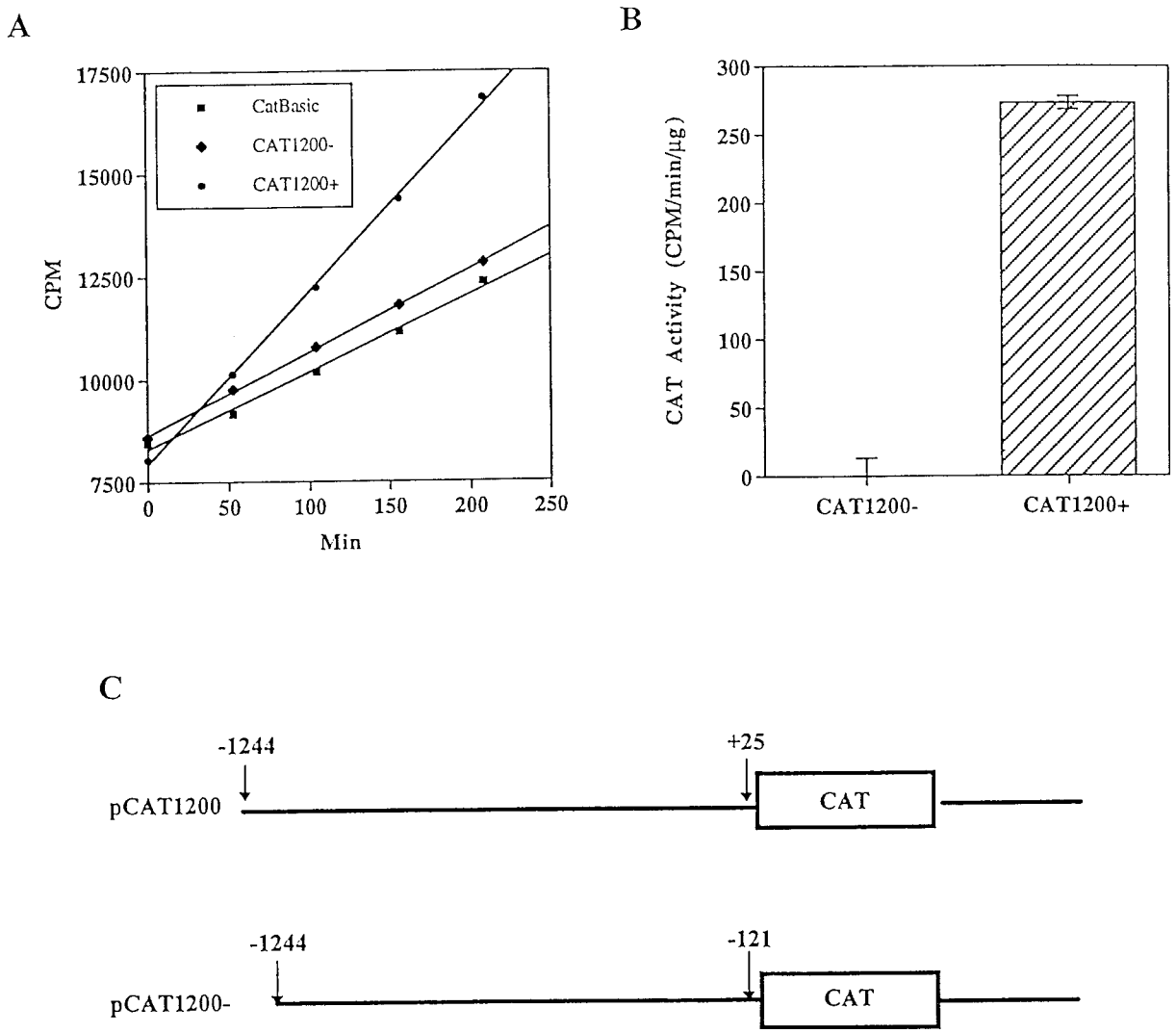

Fig. 5. Functional confirmation of the TATA box in the avian *bsp* gene promoter. **A:** Representative enzyme assay of the CAT activities of two promoter constructs containing DNA sequences between −121 to −1244 nt denoted as CAT 1200− and +25 to −1244 denoted as CAT 1200+. CAT basic which has no promoter was used as baseline. Data is presented as cpm/min/µg of protein. Linear potions of the curves are shown for each assay. **B:** Data points from triplicates were each separately used to determine the lines, and the slopes of the lines were determined. Final values were obtained by subtraction of the slope values of the base line from the experimental values. The standard deviations were calculated from the linear regression analysis of the slopes of the lines. **C:** Diagrammatic representation of the two alternate promoter constructs tested for functional activity.

cytes. A 34 fold greater activity was seen in the osteoblasts in comparison to the skin dermal fibroblasts which show a basal level of CAT activity similar to that observed for the CAT basic vector which contains no promoter. The endogenous steady state levels of BSP mRNA expression seen in these cell populations is depicted in panel B while the relationship of the promoter activity to the steady state mRNA levels is seen in panel C. As can be seen both calvaria osteoblasts and cephalic sterna chondrocytes which are capable of undergoing hypertrophic differentiation and extracellular matrix mineralization in vitro expressed high levels of the BSP mRNA. In contrast the caudal chondrocytes showed a much lower level and the dermal fibroblasts showed non-detectable levels of the expression of this mRNA. The similarity in the observed differences of the promoter's activity and the levels of mRNA between skeletal

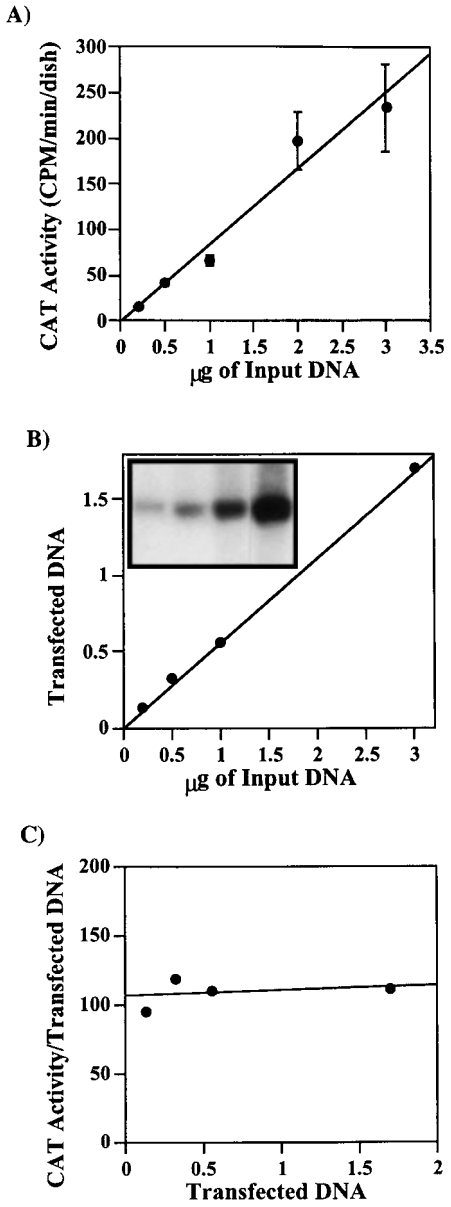

Fig. 6. Relationship of CAT activity to amount of transfected DNA. **A:** CAT activity vs amount of input DNA in the transfection. **B:** Relationship of measured amount of CAT plasmid DNA to input DNA in the transfection. Amount of transfected DNA was determined by densitometric analysis of the Southern blot of the CAT vector backbone DNA vector. **C:** Relationship of proportionality of the CAT activity vs the amount of transfected CAT vector recovered from each transfection sample.

and non skeletal cell types in which the *bsp* gene is variably expressed suggests that within the ~1200 base pairs of promoter sequence there are specific *cis* elements that are recognized in a tissue specific fashion.

From sequence analysis (Fig. 4), known silencer sequences were identified within the ~1200 base pairs of the promoter. To determine if these silencer elements play a possible role in the specific expression of the gene, nested deletions of the *bsp* promoter/CAT constructs were transfected into chicken embryonic skin fibroblast and promoter activities were assessed (Fig. 8). CAT activity did not change after the deletion of the first silencer (S1). However a dramatic 6 fold increase was observed in the pCAT-620 which contains none of the putative silencer sequences, suggesting the two silencers (S1, S2) between −978 to −620 nt may be involved in the tissue specific expression of the gene. Further deletions showed a progressive decrease in the promoter activities from that of the pCAT-620 construct but these activities were still significantly higher than the longest construct (CAT-1244) when tested in dermal skin fibroblast.

Regulation of *bsp* Expression in Osteoblasts by PTH, 1,25(OH)$_2$D$_3$ and Dexamethasome

In the last series of experiments the hormonal responsiveness of the *bsp* promoter was assessed in osteoblasts. In these experiments 10^{-8} M of PTH, 1,25(OH)$_2$D$_3$ or dexamethasome were examined. For these studies CAT assays were performed 24 h after the drug treatments and the data were calculated as a percentage of the control since in these studies identical populations of cells with a single vector were being used. The results are depicted in Figure 9. 1,25(OH)$_2$D$_3$ produced a 40% decrease in the CAT activity while dexamethasome increased the CAT by about 50%. The most striking result of the studies reported here however, was the very large fold of stimulus of the *bsp*/CAT construct in response to PTH. In these studies the CAT activity increased >5 fold after PTH treatment.

DISCUSSION

In comparison to the human and porcine *bsp* genes [Kerr et al., 1993; Kim et al., 1994], the avian gene is much more compact having only 5 exons vs. 7 exons and encompassing ~1.4 kb vs. ~6.0 kb. The major difference between the chicken genomic structure compared to the mammalian genes is the presence of an additional 5′ exon in the human gene which contains exclusively non-coding sequence and the presence of a second exon which encodes all but two amino acids of the signal peptide. In contrast, the chicken gene has a very short 5′

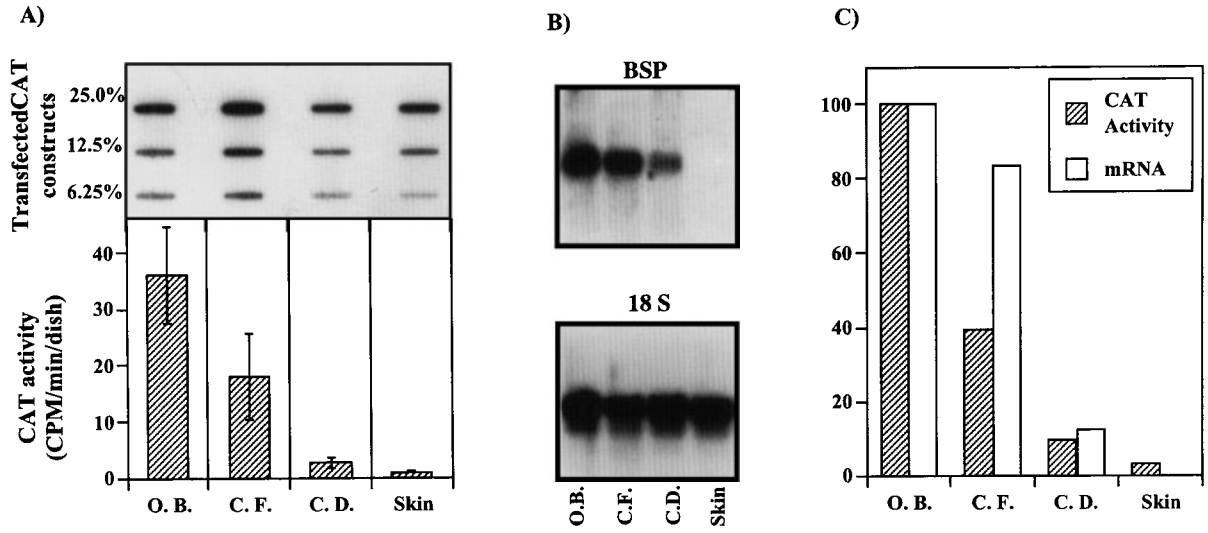

Fig. 7. Tissue specific promoter activity. **A:** Tissue specific activity of the (+25) to (−1244) nt promoter construct in four different primary embryonic chicken cell types (O.B. = osteoblast; C.F. = cephalic chondrocytes; C.D. = caudal chondrocytes; skin = skin dermal fibroblasts). Promoter activity was calculated as in Figure 6 and is expressed as cpm/min/dish. The inset in the figure depicts slot blot hybridization to the CAT vector of 25, 12.5, and 6.25% of total DNA extracted from the cell lysates used for the CAT assays. The CAT activities were then normalized to these values (see C). **B:** Steady state BSP mRNA expression in four different cell types. Upper portion of this panel depicts the Northern blot analysis obtained from 5 µg of total RNA from each of four cell types analyzed. Lower panel depicts the same blot as in the upper panel which was stripped and rehybridized to a probe of the conserved segment of the 18 S ribosomal RNA. **C:** Comparison of the promoter activities in the four cell types to the steady state mRNA levels in each cell type. CAT activities from A were normalized to relative to the amount of transfected DNA determined from the slot blots. mRNA levels were normalized values from the ratio of the BSP mRNAs to 18 S rRNA. The highest values from both experiments were set at 100%.

untranslated region lacking both the separate non-coding exon and the first intron, while the sequence encoding the signal peptide is fused with the first recognition site for casein kinase II phosphorylation. With the exception of the 5′ end the overall exon/intron structure is very similar to the mammalian genes retaining its four 3′ exons which encode the same structural domains of the protein as observed for the human gene (Fig. 1B). Of greater interest to this analysis is that the chicken *bsp* gene has a very similar exon/intron pattern with its mammalian counterparts suggesting that both proteins were originated from the same ancient gene, which emerged before the mammalian and avian evolutionary split.

In examining the promoter sequence of the avian *bsp* gene, several potential TATA elements were identified but only the most proximal one in relationship to the tsp was functional. Unlike the TATA sequence identified for the mammalian forms of this gene which are both asymmetrical and in an inverted orientation [Li and Sodek, 1993] the avian TATA element is perfectly symmetrical. In studies of the rat *bsp* promoter it was suggested that the asymmetrical TATA element has a functional role in the tissue specific expression of this gene, by selective interaction with either a distinctive TFIID complex or by altering the interaction of the TATA binding protein interactions with other *trans* factors that serve as specific activators or repressors of *bsp* expression [Li and Sodek, 1993]. While in the mammalian gene the TATA orientation may play such a function, in the avian gene the interaction of specific activators may be dependent more on the upstream *cis* elements as reflected by the sequence of the promoter. This is suggested by the presence of a Sp1 like element within the avian *bsp* promoter in contrast to the CCAAT sequences seen within the mammalian genes. Sp1 like sequences have been shown to be capable of replacing CCAAT elements as components in the basal activation of numerous genes [Dynan and Tjian, 1983; Letovsky and Dynan, 1989]. In some cases Sp1 elements have also been shown to be involved in the specific expression of genes, for instance in the human CD-14 gene an Sp1 like sequence is known to be a critical component in the activation of the genes expression in monocytes [Zhang et al., 1994]. A

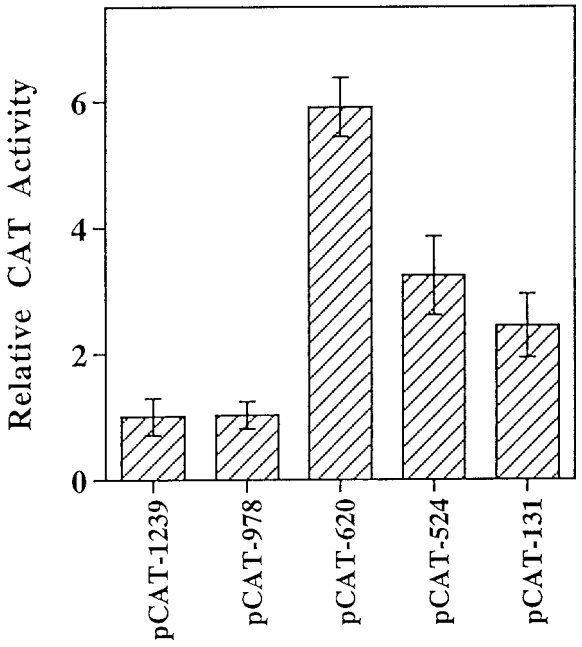

Fig. 8. CAT assay of nested deletions of the *bsp* promoter. The relative activities of progressive 5′ deletions in the *bsp* promoter in chicken embryonic skin fibroblast were assessed. The 5′ position of each deletion is denoted as in Figure 4. Values are expressed relative to the longest construct. The error bar represents the total range of variation seen in the relative values in two separate triplicate experiments.

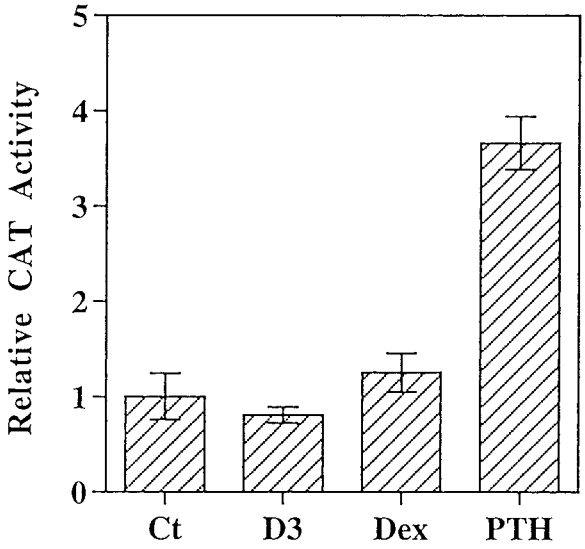

Fig. 9. Promoter response to 1,25(OH)$_2$D$_3$ Dexamethasone and PTH. Promoter activities in calvaria osteoblast cells in response to various hormones. The enzyme activities were calculated by setting the control level (no hormone treatment) to one and the change in response of each experimental treatment was measured relative to the control. DEX = 10^{-8} M Dexamethasone, PTH = 10^{-8} M PTH, and D_3 = 10^{-8} M 1,25(OH)$_2$D$_3$.

very similar proximal promoter structure containing an asymmetric TATA element and a Sp1 like sequence is also seen in the avian osteocalcin gene's proximal promoter [Simkina, Nuegebaurer, Haushka and Gerstenfeld unpublished data] and the promoters of the mammalian forms of the COL II gene [Kohno et al., 1985]. It is interesting to note that in the studies of the murine osteocalcin promoter even though a CCAAT sequence is present the *trans* acting factor that interacts with the CCAAT sequence are not competed for by consensus oligonucleotide sequences for C/EBP, CTF/NF1, or CBF/NFY, or Sp1 [Ducy and Karsenty, 1995]. It is therefore intriguing to speculate that there may be a novel transcription factor specific to skeletal tissues which is involved in the basal activation of these genes. While these authors did not find tissue specific binding of factors to region of the promoter in other studies [Hoffmann et al., 1996] these authors did observe interaction with the tissue specific factors overlapping with CCATT element. Such findings provide further evidence that their are unique factors which interact with the elements of the basal promoters of skeletal specific genes.

The widely divergent chicken *bsp* promoter contained a number of *cis* elements seen in all the other species of ECM genes examined to date which show a restricted or very elevated expression in skeletal tissues. Of greatest interest to mention are the homeobox sequences Msx2 (Hox 8.1) and the COL II silencer sequences which have been shown to be functionally active in regulating the expression of the mammalian osteocalcin gene [Towler et al., 1994; Hoffmann et al., 1994; Frenkel et al., 1993; Li et al., 1995]. The demonstrated apparent function of these elements is also suggested in the studies reported here since their deletion causes a six fold increase in the activity of the *bsp* promoter. The identification of the specific homeobox sequence engrailed-1, and its very high degree of sequence similarity for both the core element and its flanking sequences in all of the bone specific genes that were compared, suggests that this element has an important regulatory role in the expression of these genes. The recent demonstration of a novel hox protein (rHOX) isolated by its ability of binding en-1 in osteoblasts and its interaction with en-1 like sequences in the Collbox and OCbox provides further functional data indicating that this DNA element may be of importance in controlling the bone tissue specific expression

[Hu et al., 1995]. It is also of interest to note that in recent studies comparing the in vitro and in vivo activities of various genes in several different osteosarcoma cell lines, that both the COL I and BSP genes were concurrently activated upon in vivo implantation of a specific osteosarcoma cell lines suggesting that these two genes are commonly regulated [Gerstenfeld et al., 1996]. Its absence in the COL II promoter therefore may also suggest its specific role only within the osteoblast lineage. While the BSP also has a highly restricted expression in cells of the skeletal lineage, a similar sequence to one of the distinct osteoblast specific *cis*-acting elements (OSE1) that were identified, which control the expression of the mouse osteocalcin [Ducy and Karsenty, 1995] was not identified in the available sequences of either the avian or other specie of the *bsp* promoter. However, an exact sequence that is similar to OSE2 element (AACCACA) was identified in the avian bsp promoter at nt −805 to −815.

In recent studies, transgenic mice were established using a reporter construct containing 2.7 kb of the rat *bsp* promoter. The expression of this promoter construct in the transgenic animals was shown to be nearly identical to the endogenous gene [Chen et al., 1996]. It is of interest that this segment of the rat promoter contains the conserved elements identified by the sequence comparison with the ~1200 bp of the avian *bsp* promoter, since the promoter also retained perfect tissue specificity of the gene. Deletion experiments of the silencer sequences between −1239 to −620 nt of the avian *bsp* promoter suggest that these elements play a functional role in controlling the tissue specificity of this gene. The two silencer elements in this region also appeared in other genes of the skeletal lineage (Table II) and these elements appear to be general silencers which restrict skeletal gene expression in non-skeletal cell lineages. Furthermore the level of inhibition by the silencers in chicken *bsp* is similar to that of the rat COL II silencer elements [Savagner et al., 1989]. In reference to the effects of this element in the regulation of a bone specific gene, it is also of interest to note that these silencer elements also play a role in the regulation of the osteocalcin gene activity [Frenkel et al., 1993; Li et al., 1995]. Since the highest expression of *bsp*/CAT in fibroblast is still significantly lower than that in osteoblast, these data suggest the existence of a bone-specific enhancer in this segment of the promoter sequence.

The regulation of *bsp* by $1,25(OH)_2D_3$ and dexamethasone reported here were consistent with those previously reported by Oldberg et al. [1989] in which *bsp* mRNA level were shown to be increased after 24 h by dexamethasone treatment while being decreased by $1,25(OH)_2D_3$. Although long-term PTH administration has been shown to increase bone resorption, short-term or low-dose PTH treatment however increased mineral deposition within bone, suggesting that the hormone might have dual actions on both the resorption and formation sides of the coupled remodeling process. Such a suggestion of a dual function is also consistent with the results that show an inhibitory effect by PTH on collagen type I expression [Kream et al., 1993], leading to the further suggestion that PTH might target different genes at different stages of osteoblastic differentiation. Since BSP is involved in the mineralization, the induction of BSP expression by PTH treatment could be a contributory factor in new bone formation by more mature osteoblasts and play a role to the increasing bone mass, conversely its effect on collagen expression is an effect on more immature cells which would be seen during periods of resorption.

The final points of the discussion are related to the technical aspects of the approach used in these studies to assess transfection efficiency. The objective of many experiments is to identify the specific *cis* elements that control tissue specific expression. In order to compare the absolute transcriptional activity of a promoter sequence in various cell types, it is important to correct for the transfection efficiency of the DNA vector between the various cell types in which the promoter is being tested. In previous studies, transfection efficiencies have been calculated by co-transfection of a second construct with a "constitutive" promoter such as SV40, CMV, or RSV driving the expression of a different reporter such as β-galactosidase [Sambrook et al., 1989]. The activity of the reporter that is driven from the first promoter is then normalized to that of the co-transfected second reporter/promoter construct. This method however is based on the assumption that the second promoter will have the same activity in all cell types, which has been demonstrated not to be necessarily true for these constitutive promoter

[Wenger et al., 1994]. The second potential problem with this approach is that all of these constitutive promoters are very strong and even though they are transfected at a much lower concentration, their sequences may compete for limiting amounts of transcription factors with the weaker promoter that is being tested, thus potentially altering its activity. Finally when comparing a promoter's activity in different cell types in response to an exogenously added factor, the second normalizing promoter may also respond to the added perturbant. This is particularly true of viral promoters such as SV40, which are excellent responders to Ap1 activators [Angel et al., 1987]. The presence of an AP1 responsiveness by the second promoter, would confound attempts to normalize the first promoter's activity, in the presence of a factor that regulates through Ap1 activation. In the studies reported here the direct ability to easily and specifically measure the amount of transfected DNA in the transfection reactions was demonstrated and shown to have excellent correspondence to activity. These results therefore demonstrate that the direct measurement of the amount of DNA transfected into a cell may be used to directly assess transfection efficiency.

In conclusion the results presented here characterize both the conserved and divergent structural domains of bone sialoprotein gene. The major *cis* regulatory elements showing sequence similarity to the mammalian *bsp* gene include several homeobox binding sequences, several COL II silencer sequences, a half CRE element, and a VDRE element. Unlike the proximal elements in the mammalian gene which are composed of a TATA and CCAAT sequence, the CCAAT sequence appears to be replaced by a Sp1 like element in the avian gene. Approximately ~1200 nt of the avian sequence imparted a 34 fold greater activity in cells of the skeletal lineage than non-skeletal cells demonstrating that the tissue regulatory elements that control this gene's expression in skeletal cells are located in this segment of the promoter. A striking 6 fold stimulation by PTH administration was observed for this segment of the promoter in osteoblasts.

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