Journal of Cellular Biochemistry 87:458–469 (2002)

Transcriptional Regulation Restricting Bone Sialoprotein Gene Expression to Both Hypertrophic Chondrocytes and Osteoblasts

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Abstract This study identifies a *cis*-acting element that confers tissue-restricted expression to the bone sialoprotein (*BSP*) gene. Using both gain of function and loss-of function studies, we demonstrate that this element acts as a tissue specific enhancer of BSP expression in osteoblasts and hypertrophic chondrocytes but does not function in non-hypertrophic chondrocytes or fibroblasts. Furthermore, our data demonstrate that binding of this element occurs in correlation with active BSP expression. While Dlx5 has been implicated as the tissue-specific regulator of BSP expression through direct DNA binding at an element with homology to the one under study here, our results demonstrate that Dlx5 does not act as a positive regulator of BSP expression. Finally, mutational analyses of this element demonstrate that while there is homology to putative homeodomain binding elements, this site is unlikely to bind homeodomain factors including Dlx5. Thus, these studies identify an important *cis*-acting element in the BSP promoter that acts as a tissue-specific enhancer of BSP expression in both osteoblasts and hypertrophic chondrocytes. As such this is the first demonstration of a common regulatory mechanism utilized by both chondrocytes and osteoblasts for the tissue-restricted expression of the *BSP* gene. J. Cell. Biochem. 87: 458–469, 2002.

Key words: bone sialoprotein; osteoblast; chondrocyte; Dlx5

Bone sialoprotein (BSP), a phosphorylated and sulfated arginine, glycine, aspartic acid (RGD) containing glycoprotein, represents one of the major non-collagenous, extracellular matrix proteins of bone. The expression of BSP is primarily restricted to the mineralized tissues of the skeleton, specifically hypertrophic chondrocytes and mature osteoblasts [Chen et al., 1992; Yang and Gerstenfeld, 1997]. BSP is widely believed to play an important role in mineralization in skeletal tissues by regulating the nucleation of mineral and the conveyance of positional information to osteoblasts and osteoclasts through its RGD motif [Franzen and Heinegard, 1985; Gotoh et al., 1990; Yang et al., 1995]. More recent studies suggest that it may play more diverse functions in skeletal tissues since it has recently been demonstrated to possess angiogenic activity [Bellahcene et al., 2000]. Such findings along with the demonstration of its high expression in hypertrophic chondrocytes suggest the possibility that BSP may be involved in angiogenesis during endochondral bone formation or repair. Other studies suggest that when expressed ectopically by certain types of transformed cells, BSP blocks factor H mediated lysis of these cells [Fedarko et al., 2000]. Given the variety of functions for this molecule, we postulate that BSP expression may be important not only in the mineralization of both cartilage and bone but also in the linkage of chondrogenic and osteogenic processes in endochondral bone formation. As such, we believe it is important to understand

Grant sponsor: National Institutes of Health; Grant numbers: HD-22400, AR-47045; Grant sponsor: Boston University Medical Center.

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Received 14 August 2002; Accepted 15 August 2002
DOI 10.1002/jcb.10351

the regulators of tissue restricted BSP expression in hypertrophic chondrocytes and osteoblasts as well as in situations where it is ectopically expressed in specific pathologies.

A great deal of research has been undertaken to identify the regulatory molecules responsible for the restricted expression of bone genes. Previous studies have implicated several molecules as important regulators of BSP expression in osteoblasts, including Runx2 (Cbfa1, AML-3, PEBP2αA), and the homeodomain factor Dlx5 but very little data is available on the regulation of BSP expression in chondrocytes. While tissue restricted regulation of BSP by Runx2 was originally proposed based on observations from the Runx2 deficient mice which displayed diminished BSP expression, more recent studies have demonstrated that Runx2 in fact acts as repressor of the *BSP* gene [Javed et al., 2001]. Specifically, Runx2, and other related members of the Runx family, have been demonstrated to repress expression of the avian *BSP* gene in primary calvarial derived osteoblasts and Ros 17/2.8 cells in a mechanism that requires the C-terminal domain of Cbfa1 [Javed et al., 2001]. Additional reports have examined the role of Runx2 in regulating the murine *BSP* gene, however, a consensus Runx binding element was not identified in the murine *BSP* gene and forced expression of Runx2 does not modulate the expression of BSP [Benson et al., 1999]. Thus, there currently is no compelling data to support the concept that Runx2 plays an important role in the tissue-restricted expression of BSP.

Homeodomain factors, including members of both the Dlx and Msx families, have been demonstrated to be involved in the regulation of skeletal development and the bone specific gene osteocalcin [Zhang et al., 1997; Newberry et al., 1998; Benson et al., 2000]. Retroviral over-expression of Msx2 in the calvaria of chick embryos was demonstrated to produce increased proliferation while inhibiting osteoblast differentiation. Conversely, the perturbation of Msx2 through the expression of an Msx2 anti-sense construct leads to premature osteoblast differentiation and calvarial suture closure [Dodig et al., 1999]. Msx2 has also been demonstrated to repress osteocalcin expression [Newberry et al., 1998]. Mice deficient in Dlx5 also display bone related phenotypes including severe craniofacial defects as well as alterations in long bone development [Acampora et al., 1999]. Interestingly, Dlx5 has been found to enhance osteocalcin expression in osteoblasts by de-repressing the Msx2 mediated repression [Newberry et al., 1998]. Both of these regulatory actions occur through a mechanism independent of DNA binding and thought to be mediated by protein–protein interactions with the basic transcriptional machinery of the cells. However, in the case of BSP, it has been demonstrated that Dlx5 has a direct effect modulating the expression of BSP in an osteoblast specific manner through a mechanism that is dependent on DNA binding [Benson et al., 2000]. Recent studies have characterized a DNA binding site in the proximal promoter of the murine *BSP* gene that is required for tissue-specific expression in osteoblasts. This element has been proposed to regulate BSP expression by direct binding of Dlx5 to DNA at this site. This study is currently the only clear demonstration of a factor, which is able to regulate BSP expression in a tissue-restricted manner.

In this study, we have analyzed the *cis*-acting elements involved in the tissue-restricted regulation of BSP expression within hypertrophic chondrocytes as compared to mature osteoblasts, fibroblasts, and non-hypertrophic chondrocytes. For these studies, we have utilized a well characterized avian primary cell culture system in which osteoblasts, hypertrophic, or non-hypertrophic chondrocytes and skin fibroblasts are isolated from developing chick embryos. We have focused on the role of putative homeodomain factor binding sites in directing the tissue-specific activity of both the avian and human BSP promoter based on the previous demonstration that in the murine gene a similar element confers tissue-specific expression within osteoblasts. These experiments specifically examined if the previously identified *cis*-acting putative homeodomain type elements contribute to the restricted expression of BSP to both hypertrophic chondrocytes and osteoblasts thereby demonstrating a common tissue-restricted regulation of BSP in these two distinct cell types. Our results identify a *cis*-acting element that does act in both hypertrophic chondrocytes and osteoblasts and confers tissue-restricted increased BSP expression. Binding of this DNA element correlates with active BSP expression suggesting the mechanism involves direct DNA–protein interactions. As such, this is the first demonstration of a common regulatory mechanism utilized by both

chondrocytes and osteoblasts for the tissue-restricted expression of the *BSP* gene.

MATERIALS AND METHODS

Preparation of Avian Skeletal Cell Populations

Chondrocyte populations enriched in hypertrophic and non-hypertrophic cells were prepared from sterna of 17-day-old chick embryos and cultures were grown as previously described [Gerstenfeld et al., 1989]. Osteoblasts were isolated from 17-day chicken embryo calvaria by sequential trypsin and collagenase digestion as previously described [Gerstenfeld et al., 1987].

Nuclear Extracts and Electromobility Shift Assays (EMSA)

Nuclear extracts were prepared as previously described [Javed et al., 2001]. Briefly, 1×10^6 cells were washed once with ice cold PBS, trypsinized, and pelletted by centrifugation at 3,000 rpm for 5 min. The cell pellet was resuspended in 400 μl of cold NP-40 lysis buffer (10 mM Tris pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 0.5% NP-40) by gentle pipetting and incubated on ice for 10 min. The cells were pelletted by centrifugation for 30 min at 12,000 rpm and resuspended in 400 μl of cold hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl). The pellet was again collected by centrifugation at 12,000 rpm, resuspended in 50 μl of ice cold extraction buffer (20 mM Hepes pH7.9, 1.5 mM $MgCl_2$, 420 mM KCl, 0.2 mM EDTA, 20% glycerol) and vigorously rocked in the cold room for 30 min to 1 h followed by centrifugation at 12,000 rpm to remove debris. Aliquots of the supernatant containing nuclear proteins were quick frozen and stored at −80°C. Protein concentrations were determined using the Bio-Rad (Bradford) protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

Oligonucleotides used for EMSA were end labeled using ^{32}P-ATP and T4 polynucleotide Kinase. 10 μg of nuclear extract was added to a reaction mixture containing 50 mM KCl, 12 mM Hepes, 1 mM EDTA, 1 mM DTT, 12% glycerol, and 2 μg poly dIdC. In reactions containing competitor oligonucleotide, 2 pmoles of unlabeled competitor was added to this reaction. All reaction mixtures were incubated for 10 min at room temperature. Fifty femtomoles of ^{32}P-labeled probe was added to the reaction followed by an additional 20-min incubation. Reaction products were loaded onto a 5% polyacrylamide gel in 0.5 × TBE buffer and run for 1.5 h at 200 V. Gels were dried and subsequently visualized on X-Omat radiographic film (Eastman Kodak, Rochester, NY). Oligonucleotides used for the gel shift analysis include; Site A: (F) CAC TCT CCA ATT AAA TTT AGA CT, (R) AGT CTA AAT TTA ATT GGA GAG, Site B: (F) AGA CCC TCA ATT AAG TGC TGT G, (R) TCT GGG AGT TAA TTC ACG ACA C, Site C: (F) CCG TGT CCC TCT AAT TAC ATT AAA A, (R) TTT TAA TGT AAT TAG AGG GAC ACG G. All oligonucleotides are denoted in the 5′–3′ orientation.

Generation of Avian BSP Promoter Expression Constructs

Plasmid constructs containing the avian bone sialoprotein promoter from −1,244 to −100 were previously described [Yang and Gerstenfeld, 1997]. For the expression analysis of *cis*-acting element function, constructs were generated using the −100 BSP promoter construct in pCAT basic, this construct will be referred to as the BSP-100 construct. (Promega Corp., Madison, WI). The BSP −100 construct was linearized 5′ to the promoter fragment by digestion with Hind III and Pst-1 and a variety of annealed double stranded oligonucleotides representing individual DNA binding sites or mutations of these sites were subcloned into this construct in the sense orientation relative to the CAT coding sequence. All constructs were subsequently confirmed by DNA sequencing (DNA Sequencing Facility, Tufts University Core Facility, Tufts University, Boston, MA). Additional constructs were generated in the pCAT control plasmid containing the thymidine kinase promoter using a similar approach and oligonucleotides to confirm results obtained with the minimal BSP promoter. The oligonucleotide sequences are the same as used for the gel shifts but with the addition of nucleotides on each end to allow for cloning into the linearized plasmid. Element A forward orientation–(F) AGC TTG CAC TGC AGG CAC TCT CCA ATT AAA TTT AGA CTT GCA, (R) AGT CTA AAT TTA ATT GGA GAG TGC CTG CAG TGC A. Element A reverse orientation–(F) AGC TTG CAC TGC AGG CAC TCT TAA ATT AAC CTT AGA CTT GCA, (R) AGT CTA AGG TTA ATT TAA GAG TGC CTG CAG TGC A. Mutations are as indicated in the text and figure legends.

Generation of Human BSP Promoter Expression Constructs

A 2.1 kb fragment of the human *BSP* gene (−2,180 to +88) was first cloned into the Xho I site of a pGL-3 basic vector (gift from D. Benson, University of Michigan). The plasmid containing the human BSP promoter was subsequently modified using the Quikchange™ Site Directed mutagenesis Kit from Stratagene, (Cat# 200518) using a PCR reaction following the manufacturer's recommended protocol. Two human BSP promoter mutants were constructed: one was designed to convert the core of the engrailed site at −200 to −194 (5′ CAATTAA 3′) to the scrambled sequence 5′ GTCAGTC 3′ (designated element 'D') while the other changed the core of the Hox site at −403 to −412 (5′ CCTT ATTATT 3′) to the scrambled sequence 5′ CCGGCACTTT 3′(designated Site 'E'). In order to create these constructs, oligonucleotides with the sequence 5′ CTT CAT CTA AAC CTT GTC AGT CAT TCC ACA ATG CAA ACC 3′ and a reverse and complement oligonucleotide 5′ GAA GTA GAT TTG GAA CAG TCA GTA AGG TGT TAC GTT TGG 3′ were synthesized for Site A mutants while oligonucleotides with the sequence 5′ CAT CTG CTC CTT TCC CGG CAC TTT TAG TTT TTC TTT C3′ and the reverse and compliment 5′ GTA GAC GAG GAA AGG GCC GTG AAA ATC AAA AAG AAA G3′ were used for Site C mutants. The converted plasmid was transformed in competent bacteria, and after DNA isolation the integrity of the mutation within the construct confirmed by DNA sequencing.

Transfection and Expression Assays

DNA constructs were transfected into the respective cell types using the Lipofectamine reagent (Gibco, BRL, Rockville, MD) per the manufacturer's recommended protocol in Optimem media (Gibco, BRL). Transfections were carried out for 6–8 h after which cells were rinsed and fed with the appropriate media. Unless otherwise noted in the text, all transfections were carried out using 10 μg of plasmid per 100 mm cell culture dish for the chloramphenicol acetyl transferase (CAT) linked avian promoter or 1 μg of plasmid per individual well of a 6-well plate for the firefly luciferase linked human promoter. All transfections included an internal renilla luciferase linked control to normalize for individual transfection efficiencies. All samples were performed in triplicate in a minimum of three independent experiments. Cells were harvested 48 h post-transfection and analyzed for CAT activity by liquid scintillation counting or luciferase activity using the dual luciferase reporter kit (Promega).

RESULTS

DNA Binding at Site 'A' Correlates With *BSP* Gene Expression

Previous studies had identified multiple putative homeodomain DNA elements within the avian and human BSP promoters that showed sequence conservation between several different vertebrate species (depicted in Fig. 1). These elements fall into two general classes based on core sequence homologies with the avian elements A and B and human element D originally described as engrailed type elements, while the avian element C and human E originally designated as Hox 8.1 binding elements [Yang and Gerstenfeld, 1997]. The functional relationship between DNA binding activity at the three sites in the avian promoter denoted A, B, and C and the tissue restricted expression of avian *BSP* gene was initially assessed by EMSAs. EMSA analyses compared the binding patterns of nuclear extracts isolated from primary cell cultures previously demonstrated to express BSP, including osteoblasts derived from chick calvaria and hypertrophic chondrocytes isolated from the cephalic half of sterna, and non-hypertrophic cells derived from the caudal half of the sterna which do not express BSP. DNA binding was characterized according to the relative intensity and mobility of complexes between the cell types as well as their specific

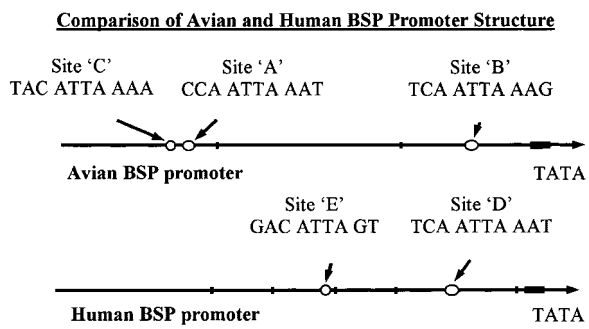

Fig. 1. Schematic comparison of the location and core sequences of the identified putative homeodomain type binding elements in the chick (top) and human (bottom) bone sialoprotein (BSP) promoters.

binding by competition with excess oligonucleotide. The resulting EMSA binding profiles fell into one of three categories: (1) binding profiles that demonstrated no change between the three cell types, (2) binding profiles which demonstrated cell type specificity in correlation with BSP expression, or (3) binding profiles which demonstrated cell type specificity not in correlation with BSP expression. Site B displayed a binding pattern of the first category with only a minor increase in binding activity observed in the chondrocyte cell extracts (Fig. 2). Sites A and C displayed binding patterns of the second type with observable complexes in both hypertrophic chondrocytes and osteoblasts but markedly decreased or undetectable binding in extracts from non-hypertrophic chondrocytes in correlation with the previously reported expression of BSP in these cell types (Fig. 2). The human element D displayed a similar binding pattern to the avian A and C elements (data not shown). In the case of element A, the intensity of binding appeared to correlate with the relative level of BSP expression in osteoblasts as compared to hypertrophic chondrocytes, i.e., osteoblast extracts displayed intense banding while hypertrophic cell extracts displayed moderate and non-hypertrophic displayed undetectable levels of complex formation (Fig. 2, panel A). A second analysis was next carried out in order to determine if the element A binding would show a temporal pattern of induction during the in vitro differentiation of the osteoblasts. As can be seen in Figure 3, the binding of this site showed a progressive increase over the time course of osteoblast differentiation suggesting that the protein factor that bound to this DNA was induced during osteoblast differentiation. Based on these correlative data, further studies were next carried out focusing on the functional activity of Site A in these cell types.

Cis-Acting Element 'A' Imparts Tissue Restricted Enhancement of Expression

The functional activity of Site A (originally demoted 5′ engrailed element) in the tissue restricted expression of the *BSP* gene was tested by cloning a single copy of this element independently in front of the minimal BSP promoter in either the forward or reverse orientation. The minimal BSP promoter contains only the proximal 100 bp connected to the chloramphenicol acetyl transferase reporter (BSP-100, CAT). This promoter construct supports only minimal promoter activity with no tissue specificity [Yang and Gerstenfeld, 1997]. Cloning of Site A into the BSP-100, CAT construct induced increased levels of BSP promoter activity in osteoblasts (>9 fold) and hypertrophic chondrocytes (~6 fold) but did not significantly

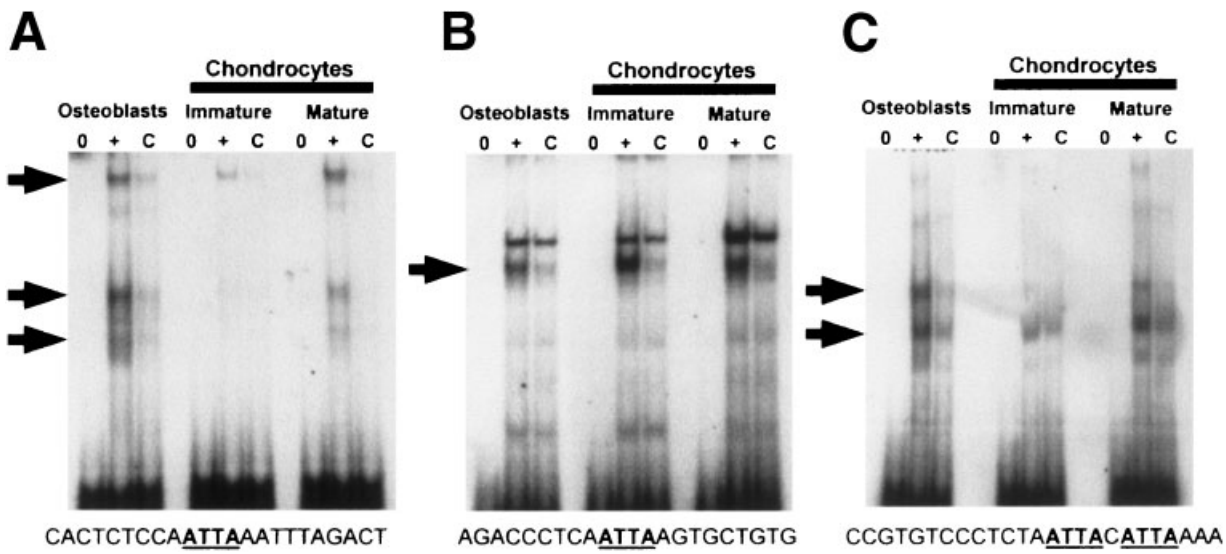

Fig. 2. Electromobility shift assay (EMSA) analysis of DNA binding correlation with the tissue-restricted expression of bone sialoprotein. **Panel A** depicts DNA binding at the A element in extracts from calvarial derived osteoblasts or sternally derived immature (non-hypertrophic) or mature (hypertrophic) chondrocytes. Arrows on left side of panel denote positions of the shifted bands. **Panel B** depicts DNA binding at the B element. **Panel C** depicts DNA binding at the C element. Lane designations; 0—no extract, + extract added, C—competition with 100 fold excess unlabelled oligonucleotide.

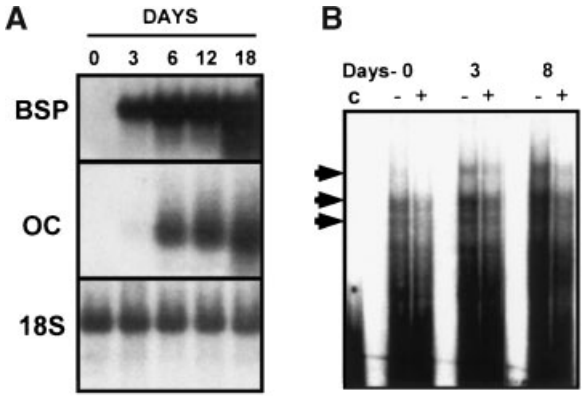

Fig. 3. Comparison of temporal profile of bone sialoprotein expression as determined by Northern blot analysis (**Panel A**) with DNA binding at element A (**Panel B**). Panel A: Northern blot analysis depicting the expression of the osteoblast marker genes BSP and osteoclacin (OC). 18s ribosomal band is shown for normalizing loading. Panel B: EMSA analysis of DNA binding activity at element A. Arrows on left side of panel denote positions of shifted bands. Days in culture is denoted at top of figure. Lane designations in panel B, C—control no extract added, – extract added, + competition with 100 fold excess unlabeled oligonucleotide.

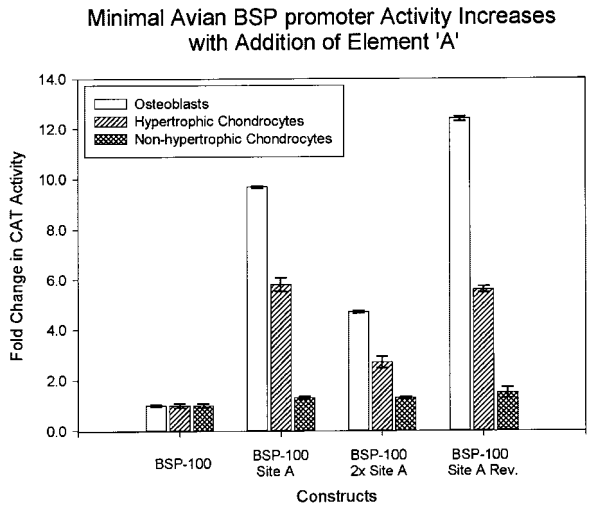

Fig. 4. Graphical representation of the fold change in relative CAT activity. Transfected primary cells include calvarial derived avian osteoblasts and sterna derived avian hypertrophic (mature) chondrocytes or non-hypertrophic (immature) chondrocytes transfected with the constructs indicated in the figure. Transfected constructs include the minimal avian BSP promoter linked to the CAT reporter (BSP-100), the minimal BSP promoter with a single copy of the ''A'' element cloned 5′ to the minimal promoter in the forward (endogenous) orientation (designated BSP-100 Site A). A similar construct including the minimal BSP promoter and two copies of the ''A'' element (BSP-100 2x Site A), and finally a construct in which the single ''A'' element has been placed in the reverse orientation to directly test for true enhancer activity (BSP-100 Site A rev.). All values are normalized to an internal renilla luciferase linked control and represent a minimum of four independent experiments with three samples per experiment.

enhance expression in non-hypertrophic chondrocytes (Fig. 4). Cloning of Site A into the same promoter construct in the reverse orientation equally imparted tissue restricted enhancement of promoter activity, demonstrating this element acts like a classic enhancer element. Interestingly, cloning of a second A motif into this construct did not significantly increase the levels of promoter activity (Fig. 4). Similarly, cloning a single Site A motif in front of a non-bone restricted promoter, thymidine kinase linked to CAT, also lead to a small but significant tissue restricted enhancement of promoter activity in hypertrophic chondrocytes and osteoblasts (1.4 and 1.3 fold, respectively) and decreased expression in the non-hypertrophic cells cultures (Fig. 5). Thus, this element was capable of conferring tissue specific regulation of expression to a heterologous promoter but at much lesser levels than when placed in context to the minimal proximal elements of the avian promoter sequence. These results demonstrate that the *cis*-acting element A (engrailed class element) acts as an important regulator of the tissue-restricted expression of BSP.

Homologous Element in the Human BSP Promoter Directs Tissue-Restricted Expression

To determine if this regulation of BSP expression is unique to the avian gene, we carried out site directed mutagenesis of the putative homeodomain elements in the human BSP promoter. The mutations generated were designed to completely abolish the core sequences of these putative elements. Expression assays of the mutated human BSP promoter linked to luciferase were compared to the wild type promoter. Mutagenesis of the D element (CAATTAA to the scrambled sequence GTCAGTC) leads to the tissue specific decrease in BSP promoter construct (Fig. 6). Mutagenesis of this element resulted in a 30% reduction in promoter activity in both osteoblasts and hypertrophic chondrocytes, while a less than 4% reduction was observed in non-hypertrophic chondrocytes (Fig. 6). In contrast, mutagenesis of element E (Hox 8.1) did not significantly alter the BSP promoter activity in osteoblasts (Fig. 6). This data demonstrates that the D element (originally classified as an engrailed type element) in the human BSP promoter acts in a functionally equivalent manner as the avian A (engrailed class) element.

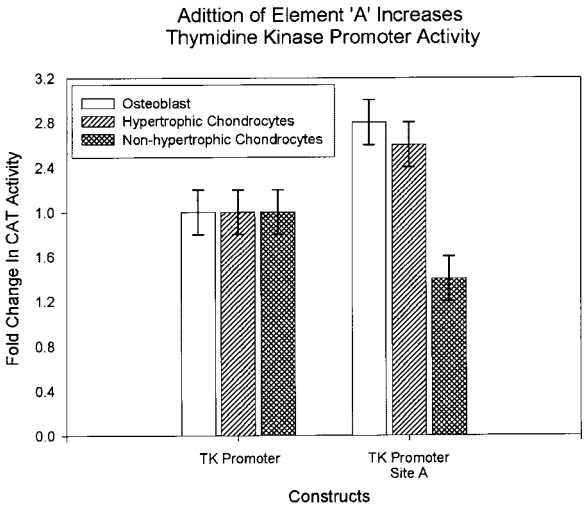

Fig. 5. Graphical representation of the fold change in relative CAT activity from primary avian osteoblasts, hypertrophic chondrocytes, or non-hypertrophic chondrocytes transfected with the designated CAT reporter constructs. Transfected constructs include the thymidine kinase promoter linked to CAT (TK promoter) and the thymidine kinase promoter with a single copy of the ''A'' element cloned 5′ to the promoter in the forward (endogenous) orientation (TK promoter Site A). All values are normalized to an internal renilla luciferase linked control and represent a minimum of three independent experiments with three samples per experiment.

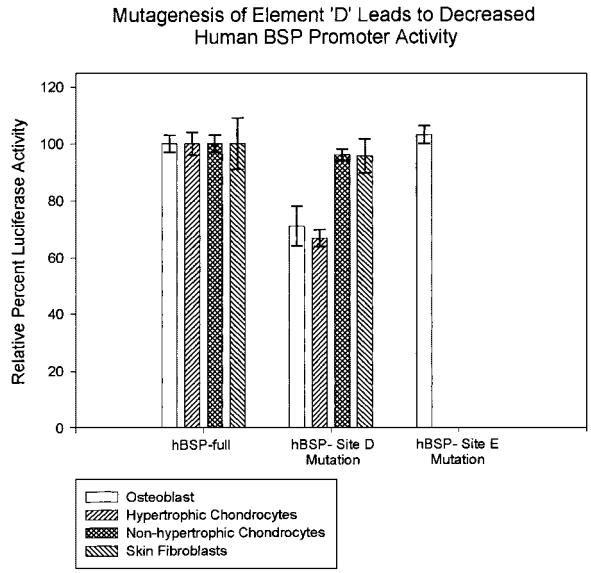

Fig. 6. Graphical representation of the relative change in luciferase activity between the full-length human BSP promoter linked to the luciferase reporter (hBSP-full) and the full-length human BSP promoter with a site directed mutation of the 'D' element (hBSP-Site D mutation) or 'E' element (hBSP-Site E mutation). Values are presented as percent relative to the full-length wild type promoter activity in each individual cell type, respectively. Transfected cells include calvarial derived avian osteoblasts, sterna derived avian hypertrophic (mature) chondrocytes or (immature) non-hypertrophic chondrocytes or skin derived fibroblasts. Transfection of the mutated E element construct was only carried out in the osteoblasts. All values are normalized to an internal renilla luciferase linked control and represent a minimum of four independent experiments with three samples per experiment.

Regulation of BSP by the Dlx5 Transcription Factor

Based on the identification of Site A as a putative homeodomain binding site with homology to that previously demonstrated to be important in the tissue-specific expression of the murine *BSP* gene in osteoblasts, mutational analyses of this site were next carried out. These studies were more specifically directed at determining if individual nucleotide changes of the core consensus motif would abolish the activity of this site in a manner consistent with previous mutational studies of homeodomain factor sequence specificity. The initial mutation was constructed to directly assess those sequences that had recently been identified in the murine BSP promoter element and had been shown to bind to Dlx5 [Benson et al., 2000]. Based on this previous study in which Dlx5 was determined to up regulate a promoter construct containing a trimer of *cis*-elements cloned 5′ to the minimal 49 bp murine, BSP promoter fragment and specific mutations of the core sequence of this element were demonstrated to abolish Dlx5 binding and regulatory activity, we generated a construct containing the same mutation. The specific mutation included the replacement of a single nucleotide in the core in addition to a nucleotide adjacent to the core (CCA**ATTA** to CC**GGTTA**, core indicated in bold with mutated nucleotides underlined). In contrast to the Benson et al. study, minimal BSP constructs containing this mutated site displayed greatly enhanced expression levels relative to the wild type element in both osteoblast and hypertrophic chondrocyte cell cultures, 12 and 4 fold, respectively, but did not alter expression levels in non-hypertrophic cells (Fig. 7). This same mutated site also imparted enhanced expression to the thymidine kinase promoter although to a lesser degree (data not shown). From this data, it was concluded that Dlx5 was not acting through this element in the avian BSP promoter.

In order to more directly assess the ability of Dlx5 to regulate BSP expression, we next carried out a series of Dlx5 over expression

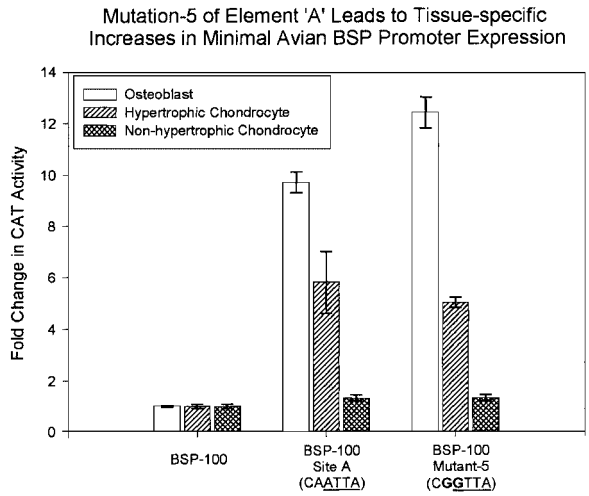

Fig. 7. Graphical representation of the fold change in relative CAT reporter activity. Transfected cells include calvarial derived avian osteoblasts and sterna derived avian hypertrophic (mature) chondrocytes or non-hypertrophic (immature) chondrocytes transfected with the constructs indicated in the figure. Transfected constructs include the minimal avian BSP promoter linked to the CAT reporter (BSP-100), the minimal BSP promoter with a single copy of the ''A'' element cloned 5′ to the minimal promoter in the forward (endogenous) orientation (designated BSP-100 Site A), and a construct in which two nucleotides have been changed in the core of the ''A'' element to generate the previously described mutation which abolishes Dlx5 binding (BSP-100, Mutant-5). Sequences of the ''A'' element core and mutated nucleotides in mutant-5 are as indicated in the figure. All values are normalized to an internal renilla luciferase linked control and represent a minimum of three independent experiments with three samples per experiment.

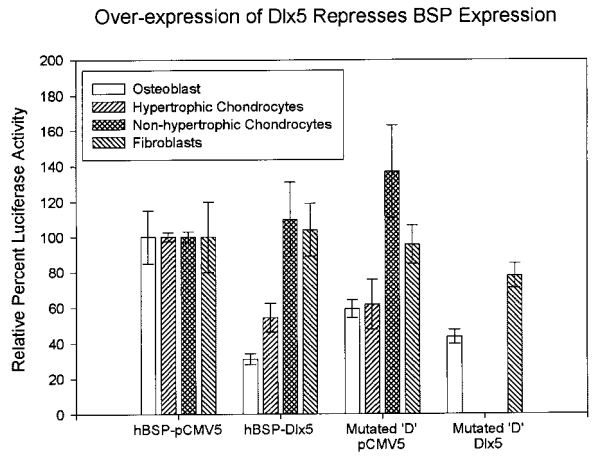

Fig. 8. Graphical representation of the relative change in luciferase activity in response to the over-expression of the Dlx5 transcription factor. Constructs include both the full-length human BSP promoter linked to the luciferase reporter (hBSP-full) and the full-length human BSP promoter with a site directed mutation of the 'D' element (mutated 'D') in combination with either over-expressed empty control plasmid (pCMV5) or pCMV5 containing the Dlx5 expression construct (Dlx5). Transfected cells types are as indicated in the figure and include calvarial derived avian osteoblasts, sterna derived avian hypertrophic (mature) chondrocytes or (immature) non-hypertrophic chondrocytes or skin derived fibroblasts. Not done are the transfections of the Dlx5 over-expression in combination with the mutated 'D' element in the chondrocytes (far right lanes). All values are normalized to an internal renilla luciferase linked control and represent a minimum of three independent experiments with three samples per experiment.

assays using both the human and avian promoters. Over expression of Dlx5 did not lead to increased BSP expression in any of the cell types tested. In contrast, Dlx5 over expression inhibited BSP expression levels in osteoblasts and hypertrophic chondrocytes and did not affect the levels of BSP expression in non-hypertrophic chondrocytes or fibroblasts (Fig. 8, avian promoter data not shown). Furthermore, site directed mutagenesis of the D element in the human BSP promoter did not significantly altered the effect of Dlx5 on BSP expression suggesting that Dlx5 does not regulate BSP through DNA binding at the D element. Taken together, the mutagenesis and over expression data clearly demonstrate that Dlx5 does not act as an inducer of BSP expression but rather suggest that Dlx5 may act as a tissue-restricted inhibitor of BSP expression. Thus, this data is inconsistent with the regulatory function of the element identified in these studies.

Mutagenesis of the A Element

Due to the unexpected results from the initial mutation described above (CCAATTA to CCGGTTA), four additional mutations to this element were generated based on other previously reported studies detailing the binding specificity of homeodomain binding factors [Catron et al., 1993]. For these studies, a series of oligonucleotides containing mutations across the core ATTA (TAAT) motif were generated and cloned in front of the minimal BSP-100, CAT construct (Fig. 9). These constructs were then transfected into osteoblast cells to determine the relative activities of the individual constructs. Of the four mutations only the fourth mutation (ATTA to ATGG mutation of the core) completely abolished the site's enhancer activity (Fig. 9). This is in contrast to the third mutation that produced an enhancement of expression despite its similarity to the fourth (ATTA to GGTA vs. ATGG). The second mutation, a single nucleotide change (ATTA to

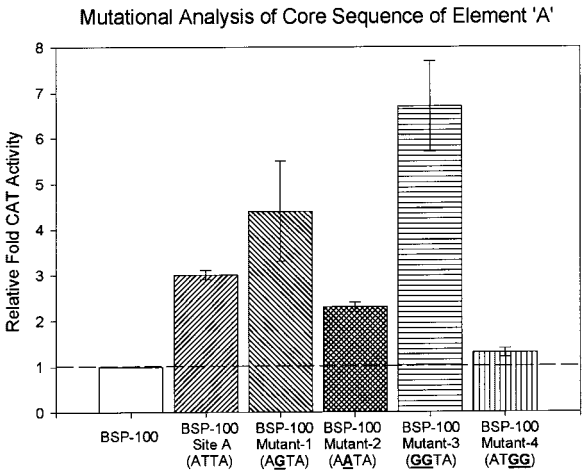

Fig. 9. Graphical representation of the fold change in relative CAT reporter activity in transfected primary avian calvarial osteoblasts. Transfected constructs include the minimal avian BSP promoter (BSP-100), the minimal avian BSP promoter construct containing a single wild type "A" element (BSP-100 Site A), and four independent minimal BSP promoter constructs containing mutations across the core sequences of the "A" element. Specific nucleotide changes in core sequence are indicated in the figure. All values are normalized to internal renilla luciferase linked controls and represent a minimum of three independent experiments with three samples per experiment.

AGTA), reduced the enhancement of BSP expression while the first mutation also enhanced the expression levels of the promoter construct. These analyses demonstrate an interesting asymmetry within the core of this element and suggest that factors other than classic Homeodomain factors are binding this site.

DISCUSSION

The majority of research into the tissue-restricted expression of skeletal genes has focused on the transcriptional regulators of genes within osteoblasts. Considerable new information has emerged that demonstrates that hypertrophic chondrocytes and osteoblasts share many of the same phenotypic features and that there is some form of functional relationship between chondrogenic and osteogenic cell differentiation [Gerstenfeld and Shapiro, 1996]. These data then suggest that further research is needed to identify both common and divergent regulators that both restrict gene expression in skeletal cells in general and specifically in hypertrophic chondrocytes in comparison to osteoblasts. Interesting new potential roles for BSP in bone development have underscored the importance of understanding the tissue-specific regulation of this specific gene. Unlike osteocalcin that is almost exclusively restricted in its expression to osteoblasts, BSP is expressed in both calcified cartilage and bone. Specifically, BSP expression is restricted to hypertrophic chondrocytes and osteoblasts [Chen et al., 1992; Yang and Gerstenfeld, 1997]. Mineralization of these tissues is important in the initial formation of bone as well as post-natal bone repair. Recent reports have suggested that in addition to a central role in mineralization, BSP may also play a role in vascularization such as occurs during endochondral bone development where the vascularization of calcified cartilage and the associated influx of osteoprogenitor cells is essential for the replacement of cartilage with new bone [Bellahcene et al., 2000]. While a great deal of effort has been directed at understanding the restricted expression of BSP to osteoblasts, no research has focused on the expression of this same gene in hypertrophic chondrocytes or in identifying common molecular mechanisms that regulate genes in both tissues.

Research into the regulation of the tissue-restricted expression of BSP in osteoblasts has focused on a limited number of specific factors originally determined to regulate osteocalcin expression. Osteocalcin expression is restricted to cells of the osteoblastic lineage and is considered a marker of relatively mature osteoblasts. Originally, many researchers expected that osteocalcin and BSP would be similarly regulated in osteoblasts due to their restricted expression in this cell type. Several recent studies have now demonstrated that the regulation of BSP and osteocalcin are not similar [Benson et al., 2000; Javed et al., 2001]. Specifically, Runx2, and other related members of the Runx family, have been demonstrated to repress expression of the avian *BSP* gene in primary calvarial derived osteoblasts and Ros 17/2.8 cells in a mechanism, which requires the C-terminal domain of Cbfa1 [Javed et al., 2001]. These results are in contrast to the positive regulation of osteocalcin by Runx2 through the well described OSE-2 element [Ducy et al., 1997; Xiao et al., 1997]. Additional reports have examined the role of Runx2 in regulating the murine *BSP* gene. However, no consensus Runx binding elements were identified in the murine *BSP* gene and forced expression of Runx2 did not modulate the expression of BSP [Benson et al., 1999]. Thus, there currently exists no com-

pelling data supporting the concept that Runx2 plays an important role in the tissue-restricted expression of BSP. In a similar manner, Dlx5 has been demonstrated to act as a tissue specific enhancer of BSP expression in osteoblastic cells in a mechanism which involves direct DNA binding while the regulation of osteocalcin by Dlx5 occurs by a mechanism independent of DNA binding and is primarily achieved through the perturbation of Msx2 inhibition instead of an enhancement, although both mechanisms ultimately lead to increased expression [Newberry et al., 1998; Benson et al., 2000].

It is important then to recognize that BSP and osteocalcin expression patterns temporally differ during osteoblast differentiation and skeletal development with BSP expression initiated earlier in osteoblast differentiation. Finally, different mechanisms of regulation should not be unexpected considering the differing functional roles these two molecules play in bone formation. BSP primarily plays a role in the early stages of mineralization while osteocalcin is speculated to be primarily involved in later stages of bone formation and predominantly functions in bone remodeling and osteoblast interactions with osteoclasts [Ducy et al., 1996; Javed et al., 2001]. Thus, the differential regulation of these two skeletally expressed genes is consistent with the differing temporal and functional activities of these two molecules.

These studies are focused on identifying common molecular regulators of BSP expression in both osteoblasts and hypertrophic chondrocytes. We have identified a unique *cis*-acting element with homology to putative homeodomain binding elements in the distal portion of the avian BSP promoter that acts as a tissue restricted regulator of BSP expression in both hypertrophic chondrocytes and osteoblasts, the two primary cell types that express BSP. In addition, this study demonstrates that a homologous element located more proximally in the human BSP promoter (D) acts in functionally equivalent capacity. Both gain of function studies in which addition of a single copy of this 'A' element is demonstrated to be capable of conferring this tissue-restricted regulation to both a minimal BSP promoter (Fig. 4) as well as a non-bone specific thymidine kinase promoter (Fig. 5) and loss of function studies in which the functionally equivalent element is mutated in the human BSP promoter (Fig. 6) demonstrate a role for this element in the tissue restricted regulation of BSP expression to hypertrophic chondrocytes as well as osteoblasts. It is interesting to note that while the avian and human elements confer the same tissue restricted regulation, they are located in distinctly different positions in their respective promoters with the avian promoter element residing in a relatively distal location (−966 to −949) compared to the element in the human promoter which is localized in the proximal promoter (−205 to −190) in a position similar to homologous elements in both the rat and mouse genes (Fig. 1) [Benson et al., 1999, 2000]. The human promoter does not have a second distal located element. While the avian gene does have a second highly homologous element in the proximal position (refer to Fig. 1), this element does not display the same restricted DNA binding pattern demonstrated for the distal element (Fig. 2, panel B). These differences are presumably the result of evolutionary divergence between birds and mammals.

One unique aspect of the element identified within the BSP promoter is the correlation between DNA binding complex formation, as determined by EMSA, and the expression of BSP. Binding at the 'A' element correlates with both the tissue restricted expression profile of BSP in both osteoblasts and hypertrophic chondrocytes (Fig. 2) as well as the temporal profile of induction of BSP expression during osteoblast mineralization (Fig. 3). This correlation clearly suggests that regulation through this element requires direct DNA binding by the factor(s) involved. This conclusion is further supported by the results demonstrating that site directed mutagenesis of this element leads to diminished BSP expression (Fig. 6). Furthermore, the relative similarity in size of complexes formed between hypertrophic chondrocytes and osteoblast cell extracts suggests the same factor(s) bind this element in both chondrocyte and osteoblast cells, although this has yet to be definitively demonstrated (Fig. 2). These observations are interesting in light of the data regarding homeodomain factor regulation of osteocalcin gene expression. Both Dlx5 and Msx2 have been demonstrated to regulate osteocalcin expression in osteoblast cells, however, this regulation has been demonstrated to be independent of DNA binding [Zhang et al., 1997; Newberry et al., 1998]. In the case of BSP regulation, recent studies have demonstrated that Dlx5 regulates the murine *BSP*

gene via a mechanism that requires DNA binding [Benson et al., 2000]. Thus, there appears to be a distinct difference between the mechanisms utilized by this molecule (Dlx5) to regulate these two genes. Our data supports the conclusion that regulation of *BSP* gene expression through this element occurs via a DNA binding dependent mechanism.

Where our data differs from the previous study on the regulation of BSP is in the identity of the factor binding at this element. This study through mutational analyses demonstrates that Dlx5 is not the factor binding this element. Specifically, we have generated mutations within the core sequence of this element which have been demonstrated by others to abolish the specific binding of Dlx5 as well as mutations previously demonstrated to abolish most, if not all, homeodomain factor binding (Figs. 7 and 9) [Catron et al., 1993; Benson et al., 2000]. In addition, we have directly assessed the ability of Dlx5 to regulate BSP expression through a series of over expression assays in a variety of cell types and determined that Dlx5 does not act as a positive regulator of BSP expression (Fig. 8). In fact, our data demonstrate that Dlx5 acts as a tissue-restricted negative regulator of BSP expression in osteoblasts and hypertrophic chondrocytes. The explanation for the difference between our data and that previously reported is likely to lie in the DNA constructs used and/or the cell system in which these constructs were tested. The previous study utilized an artificial promoter construct in which a trimer of *cis*-elements were cloned 5′ to a minimal 49 bp murine BSP promoter and tested the ability of Dlx5 to activate this construct in a cell line which does not endogenously express BSP, COS7 cells [Benson et al., 2000]. In our studies, we have utilized the intact full-length human promoter and tested the ability of Dlx5 to induce expression in several primary cell lines that express BSP endogenously (osteoblast and hypertrophic chondrocytes) as well as those that do not (non-hypertrophic chondrocytes and fibroblasts). In addition, we have compared the ability of Dlx5 to regulate the intact BSP promoter compared to a construct in which the *cis*-element has been mutated in the context of the full-length promoter (Fig. 8). Thus, our data does not support the conclusion that Dlx5 is the positive tissue-restricted regulator of BSP expression interacting at this *cis*-element and further suggest that the factor(s) binding this element are not homeodomain factor(s).

In summary, this report supports the previous work demonstrating that a *cis*-acting element with homology to previously described engrailed type DNA binding elements in the bone sialoprotein promoter acts as a tissue specific enhancer of BSP expression in osteoblasts. This study extends these observations and demonstrates that this same element regulates the tissue-restricted expression of BSP to hypertrophic chondrocytes. As such, this report represents not only the first demonstration of an element involved in the tissue-restricted expression of BSP to hypertrophic chondrocytes but also the first demonstration that this gene is regulated by a common regulatory mechanism in the cells of both the osteoblastic and chondrogenic lineages.

ACKNOWLEDGMENTS

The authors thank Johanna Cruceta for assistance with the primary cell cultures and Dr. Jane Lian and Dr. Gary Stein (University of Massachusetts Medical Center, Worcester, MA) for kindly providing the Dlx5 expression construct. This work was supported in part by funding from the National Institutes of Health (HD-22400 and AR-47045 to L.C.G.) and institutional support from the Boston University Medical Center.

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