RESEARCH COMMUNICATION

Cloning and characterization of the rat bone sialoprotein gene promoter

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To study the transcriptional regulation of the rat bone sialoprotein (BSP) gene, the nucleotide sequence of a ~1 kb *HindIII/KpnI* subfragment from a genomic clone containing the 5' flanking sequence, exon 1 and part of intron 1 was determined and the transcription start site defined. This region includes an inverted TATA element (nt -24 to -19), an inverted CCAAT box, a homeobox-binding site, a putative 1,25-dihydroxyvitamin D₃ response element (VDRE) sequence overlapping the inverted TATA sequence, and a novel 18 nt palindrome that may control the tissue-specific transcription of the BSP gene. The shortest

promoter sequence capable of directing bacterial chloramphenicol acetyltransferase reporter gene expression included the inverted TATA element and the inverted CCAAT box. However, the promoter activity was down-regulated by 1,25-dihydroxyvitamin D₃, indicating that the unique VDRE-like sequence overlapping the TATA element is functional. Thus the rat BSP gene promoter is characterized by novel *cis*-acting elements that may be involved in hormone- and tissue-specific regulation of transcription.

INTRODUCTION

Bone sialoprotein (BSP) is a highly sulphated and phosphorylated glycoprotein that is present almost exclusively in the mineralized connective tissue matrices of bone, dentine and cementum [1–8]. The cDNA of rat BSP has been cloned and found to encode a 33600 Da secreted protein of 320 residues [5]. The protein is characterized by its ability to bind strongly to hydroxyapatite, presumably through two or three stretches of poly(glutamic acid), and to mediate cell attachment through an RGD motif [9–11]. In rat bone, BSP is specifically expressed by fully differentiated osteoblasts, with especially high levels of expression observed at sites of *de novo* bone formation [8,12,13]. Moreover, the expression of the BSP gene is developmentally regulated [14]. Expression of the BSP gene by adult rat bone marrow cells *in vitro* is induced by the synthetic glucocorticoid dexamethasone in association with bone formation [15]. In contrast, BSP gene expression is down-regulated by the osteotropic hormone 1,25-dihydroxyvitamin D₃ [16], which promotes bone remodelling. Consequently, BSP is believed to function in the initial formation of the mineralized bone matrix [8,17].

Since the elucidation of the molecular mechanisms that regulate the tissue-specific expression of the BSP gene can provide important insights into the understanding of bone and tooth development, as well as of metabolic and congenital diseases of mineralized tissues, we have isolated a genomic clone that includes the promoter region of the rat BSP gene. Characterization of the promoter has revealed novel regulatory elements that may control the tissue-specific and developmental expression of this protein. This paper describes the first characterization of a BSP gene promoter.

MATERIALS AND METHODS

Genomic library screening

cDNA probes used for genomic library screening were generated

by PCR, using a rat BSP cDNA clone as template. Specific regions of rat BSP cDNA were amplified, with *EcoRI* tails added to the primers, cloned into pT7T3 18U, and radiolabelled with [α -³²P]dCTP by oligolabelling (T7QuickPrime Kit, Pharmacia). A rat genomic library prepared from rat testis DNA and assembled into λ phage DASH II vector (Stratagene) was screened by plaque hybridization to the full-length cDNA probe, including the 5'-untranslated region (5'-UTR) and the entire BSP coding sequence [5], according to the supplier's (Stratagene) instructions. Positive clones were further selected using cDNA probes derived from a 5'-fragment and a 3'-fragment (nucleotides -70 to +56 and +829 to +960 respectively relative to the ATG start codon) [5].

Genomic mapping and DNA sequencing

From a clone (λ 4A) encoding the 5'-region of the rat BSP gene a ~5 kb *XbaI* fragment was digested with various restriction enzymes. The sub-fragments were separated by agarose gel electrophoresis and directly hybridized with the 21-mer ³²P-end-labelled oligonucleotide (RBSP5.1) (see Figures 1a and 1b) to provide a genomic map [18]. Relevant DNA fragments were excised with the appropriate restriction enzymes and subcloned into pT7T3 18U. Sequencing was performed on both DNA strands using the Sanger dideoxy chain termination technique (T7Sequencing Kit, Pharmacia). The nucleotide sequences were analysed in the Transcription Factors Database using the University of Wisconsin Genetics Computer Group Program.

Primer extension analysis

A 20-mer ³²P-end-labelled oligonucleotide (50000 d.p.m. per reaction) complementary to nucleotides 37–56 downstream of the ATG start codon (RBSP3.1) [5] was hybridized overnight to 10 μ g of total fetal rat bone RNA at 30 °C. Nascent cDNA was synthesized by avian myeloblastosis virus reverse transcriptase

Abbreviations used: BSP, bone sialoprotein; CAT, chloramphenicol acetyltransferase; CRE, cyclic AMP response element; UTR, untranslated region; VDRE, 1,25-dihydroxyvitamin D₃ response element.

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according to Frederickson et al. [19] and the ^{32}P -labelled cDNA generated was analysed on an 8 % polyacrylamide/8 M urea gel.

Chloramphenicol acetyltransferase (CAT) plasmid construction

The transient expression vector pCAT-Basic (Promega) containing the bacterial CAT reporter gene, but lacking a promoter and enhancers, was used to test for BSP gene promoter activity. Using the isolated rat BSP genomic fragment as template, 5'-deletion mutants were generated by PCR with *HindIII* or *SalI* tails added to the primers, and cloned into the *HindIII*/*SalI* sites of the pCAT-Basic vector. The identities of the deletion mutants were confirmed by nucleotide sequencing on both DNA strands.

DNA transfection and CAT analysis

The osteoblast cell lines ROS17/2.8 and RCJ3.1 were grown in α -minimal essential medium containing 10 % (v/v) fetal bovine serum and seeded 24 h prior to transfection at a density of 4×10^5 cells per 100 mm dish. Transfections were performed by the calcium phosphate precipitation technique [18,20], using 10 μg of BSP-CAT fusion plasmid DNA for each dish. Normalization of CAT enzyme activity for transfection efficiency was determined by co-transfection with 2 μg of pCH110 (Pharmacia) containing the *lacZ* gene driven by the simian virus 40 promoter. Cells were harvested at ~ 60 h after transfection, and cell extracts were prepared by repeated freeze-thawing. Some transfected cells were treated with 100 nM 1,25-dihydroxyvitamin D_3 for 60 h after transfection to test the responsiveness of the BSP gene promoter. Either liquid scintillation counting or t.l.c. was employed to determine the CAT activities, according the manufacturer's (Promega) instructions.

RESULTS AND DISCUSSION

To characterize the *cis*-acting sequences involved in regulation of the transcription of the rat BSP gene, a rat genomic library was screened for clones hybridizing with a full-length rat BSP probe. Seven independent λ clones were obtained from the initial screen of 1×10^6 phage plaques. One clone ($\lambda 4\text{A}$) which hybridized to a 5'-end cDNA probe, but not to a 3'-end probe, was digested with *XbaI*. A ~ 5 kb *XbaI* fragment from $\lambda 4\text{A}$, recognized by the 21-mer oligonucleotide (RBSP5.1), was subcloned into the *XbaI* site of vector pT7T3 18U (clone pGRBSPX31) and a restriction map of the insert was determined using the following enzymes: *HindIII*, *HincII*, *KpnI*, *SacI*, *SalI* and *XbaI* (Figure 1a). Since this insert was found to include ~ 3 kb of the 5'-flanking region, a ~ 1 kb *HindIII*/*KpnI* fragment from pGRBSPX31 that hybridized to oligonucleotide RBSP5.1 was subcloned for further analysis (clone pGRBSPHK7). The complete nucleotide sequence of the insert of pGRBSPHK7 was determined for both DNA strands (Figure 1b) and numbered relative to the transcription start site (see below). The nucleotide sequence of the insert of pGRBSPHK7 contained a 63 bp region which is identical to the 5'-UTR of the rat BSP cDNA except for one G-T mismatch (+19 from transcription start site) [5], and includes a consensus splice donor site (AG:GTAAGGT), defining the 3'-boundary of exon 1.

The transcription start site of the rat BSP gene was determined initially by primer extension mapping. Total fetal rat calvarial RNA, shown previously to express abundant BSP transcripts [13,14], was analysed, using total fetal rat liver RNA and yeast

tRNA as controls. Precise localization of the initiation sites of BSP transcripts was carried out by comparison of the primer extension products with an adjacent Sanger sequencing ladder. Three start sites were found (Figure 2), the major start site being designated in the nucleotide sequence as +1 (Figure 1b). The position of the major transcription start site was subsequently confirmed by RNAase protection mapping (results not shown).

An examination of the 5'-flanking sequences of the rat BSP gene revealed a number of sequences resembling consensus eukaryotic *cis*-acting elements (Figure 1b): an inverted TATA element (TTTATA) at -24 to -19, an inverted CCAAT box (ATTGG) at -50 to -46, a cyclic AMP response element (CRE) (TGACGTCG) at -75 to -66, a homeobox-binding site (TCAATTAAAT) at -194 to -185, a potential AP-1 site (TTATTCA) at -143 to -137, a putative AP-2 site (GGCAGCCCC) at -447 to -440, and a sequence (AGGGTTtatAGGTCA) overlapping the inverted TATA element that resembles a 1,25-dihydroxyvitamin D_3 response element (VDRE). In addition, a novel 18-nucleotide perfect palindrome was located at -453 to -436.

The close proximity (within 5 bp) of the major and minor transcription start sites (Figure 2) supports the existence of a TATA box that positions the RNA polymerase II for accurate transcription initiation [21,22]. Notably, a TATA-like sequence was located at -24 to -19 in the rat BSP gene promoter. However, this putative TATA box is in a reversed orientation on the complementary strand (inverted). The TATA box is believed to determine the transcription start site, direct the formation of a preinitiation complex, and mediate the activity of upstream activator elements [22-25]. In addition, the TATA box is thought to define the direction of transcription because of the inherently asymmetrical nature of most TATA sequences [26]. However, the hypothesis that the TATA box is involved in determining the direction of transcription is still under debate. By using synthetic promoters and an *in vitro* transcription system, O'Shea-Greenfield and Smale [27] have recently shown that both normal and inverted TATA sequences can drive transcription from similar initiation sites and in the same direction, although transcription from an inverted TATA box is reduced by 7-fold. In addition, a functional inverted TATA box located 15 nucleotides downstream of the transcription start site has been described in the adenovirus IVa2 promoter [28]. Our study has identified the first inverted TATA element located at the position of a TATA box for a eukaryotic gene. Should this putative TATA element prove to be functional in promoting downstream transcription of the rat BSP gene, this would support the contention that the orientation of transcription is not dependent upon the orientation of the TATA box. Notably, the putative inverted TATA box together with the inverted CCAAT box are conserved at corresponding positions in the recently isolated human BSP gene promoter (R. Kim, H. S. Shapiro, J. J. Li, J. L. Wrana and J. Sodek, unpublished work). Since the spatial and temporal expression of the BSP gene is highly restricted, and heterogeneous TATA boxes may function as a selective element to determine tissue-specific gene expression by interaction with distinctive TFIID complexes [25,29], it will be of interest to investigate the significance of the inverted TATA sequence in the BSP gene.

To test promoter activity we initially used an osteoblastic cell line, UMR106-01, that expresses the BSP gene constitutively [30] for transient transfection. However, the transfection efficiency for UMR106-01 cells was insufficient to test for promoter activity. Consequently, five BSP-CAT fusion constructs were transiently transfected into another osteoblastic cell line, ROS17/2.8, and examined for CAT enzyme activity (Figure 3). The shortest sequence (-116 to +60) capable of directing high-level (10.6

Figure 1 (a) Restriction enzyme map of the 5'-flanking region of the rat BSP gene, and (b) nucleotide sequence of the promoter region of the rat BSP gene

(a) A ~ 5 kb *XbaI* fragment from genomic clone λ 4A that includes ~ 3 kb of 5'-flanking region was subcloned and designated as pRBSPX31. The position of the first exon is represented as an open box and restriction sites are indicated. The transcription start site, as determined in Figure 2, is indicated as +1. The position of hybridization probe RBSP5.1 used for in-gel Southern blotting is also illustrated. (b) A ~ 1 kb *HindIII/KpnI* fragment, as illustrated in (a), was subcloned and sequenced. The major transcription start site (▼), determined as shown in Figure 2, is numerically designated as +1. Minor start sites are also presented (▽). The splice donor (S.D.) site is indicated, and the partial 5'-UTR sequence of rat BSP cDNA [5] is shown in italics for comparison. The site of probe RBSP5.1, used for in-gel Southern blotting, is indicated by a broken underline. Consensus sequences for various *cis*-acting elements discussed in the text are also depicted. The positions of the 5'-ends of the chimaeric gene constructs used in transient expression studies are indicated by the arrowheads, whereas the 3'-end is indicated at +60.

times relative to the vector control) CAT expression included the inverted TATA element, the inverted CCAAT box and the CRE. Similar results were found when the same constructs were transfected into a non-transformed rat bone cell line, RCJ3.1 (results not shown). The existence of a CCAAT box can ensure

optimum promoter activity, and the CCAAT box is operative in either orientation [22,24]. Moreover, the CCAAT element is also believed to be a main target for regulation of specific genes, as exemplified by the expression of multiple CCAAT transcription factors (CTF) in a tissue-specific and development-specific pat-

Figure 2 Determination of the start site of transcription

The primer-extended products obtained using RBSP3.1 as a primer were co-electrophoresed with Sanger dideoxy sequencing reaction products to determine the precise start sites. The longest, and major, product was 154 bp and, therefore, is designated as +1 as shown in Figure 1(b). Minor start sites were evident at 151 bp and 150 bp. Fetal rat calvarial RNA (lane 1) was used as a template for the BSP transcripts. Negative controls are fetal rat liver RNA (lane 2) and yeast tRNA (lane 3).

tern [31], while the CCAAT-enhancer binding protein (C/EBP) can act as a differentiation switch for execution of a specialized phenotype [32]. The sequence upstream of the CCAAT box appears to contain a negative regulatory element(s), since inclusion of the DNA sequences upstream of -116 in the construct effectively suppressed transcription in ROS17/2.8 cells (Figure 3). Inspection of this region revealed a sequence (CACCTCT at -408 to -402) that closely resembles the suppressing element (CACCTCC) found in the rat collagen II gene [33]. Of interest is the fact that similar suppression sequences are also present in the promoter of the osteopontin gene [34].

Figure 3 Promoter activity analysis by 5'-deletion

Five fragments of the rat BSP gene 5'-flanking region were generated by PCR using specific oligonucleotide primers with *Hind*III or *Sal*I tails (shown in Figure 1b), cloned in the 5' to 3' orientation between the *Hind*III and *Sal*I sites of pCAT-basic plasmid, and designated pCAT1 to pCAT5. The identities of the deletion mutants were verified by nucleotide sequencing. The bar values represent means $\pm$ S.D. from seven independent transfection experiments, each performed with replicates as measured by liquid scintillation counting CAT assays and normalized to β -galactosidase activity. pCAT-control (pCATC), which has both the promoter and enhancer of simian virus 40, was used as a positive control, whereas pCAT-basic (pCATB), which lacks promoter and enhancer, was used as a negative control. The relative levels of CAT expression to those of pCATB from each construct are illustrated on the right.

Figure 4 Down-regulation of rat BSP gene promoter by 1,25-dihydroxyvitamin D₃

ROS17/2.8 cells were transfected with pCAT3 (as in Figure 3) and cultured in the presence (lane D+) or absence (lane D-) of 1,25-dihydroxyvitamin D₃. pCAT-basic (lane B) transfection was used as a negative control, and pCAT-control (lane C) transfection as a positive control. The data shown represent a typical result of t.l.c. CAT assays obtained from three independent transfection experiments, each performed with replicates. Acetylated and unacetylated forms of [¹⁴C]chloramphenicol were separated on silica plates and exposed to X-ray film. The pCAT3 promoter activity is suppressed by $28.4 \pm 1.4\%$ (mean $\pm$ S.E.M.), as measured by liquid scintillation counting. Neither 1,25-dihydroxyvitamin D₃ nor ethanol vehicle affected the expression of the control vectors (results not shown).

The observation that significant promoter activity was obtained in osteoblastic cells, in which the BSP gene is not expressed, indicates that the tissue-specific expression of the BSP gene involves upstream sequences which may act in concert with elements in the promoter region. Thus the suppressed transcription by upstream sequences could reflect the phenotype of the transfected ROS17/2.8 cells. Notably, a 10 bp consensus homeobox-binding site (TCAATTAAAT) that is recognized by the *engrailed* (*en*) and the *fushi tarazu* (*ftz*) proteins, and a unique 18-nucleotide palindrome which could also be involved in cell-specific expression of BSP, are found within this region. In this regard, homeobox proteins are thought to be key regulators of embryonic development [35], and the same homeobox-binding

site is conserved in the human BSP gene promoter at the corresponding position (R. Kim, H. S. Shapiro, J. J. Li, J. L. Wrana and J. Sodek, unpublished work).

A VDRE-like sequence was found overlapping the putative inverted TATA box, located at -28 to -14. This sequence (AGGGTTtatAGGTCA) is very similar to the consensus VDRE (AGGTCAnnnAGGTCA) [36]. Notably, in the promoter of the human osteocalcin gene the glucocorticoid receptor binds to a sequence overlapping the TATA box and provides a mechanism for suppressing osteocalcin transcription by glucocorticoids [37]. To determine whether the VDRE-like sequence in the rat BSP gene promoter is involved in the down-regulation of BSP gene transcription by 1,25-dihydroxyvitamin D₃, transiently transfected ROS17/2.8 cells were treated with 100 nM 1,25-dihydroxyvitamin D₃. Compared with vehicle control treatment (ethanol), 1,25-dihydroxyvitamin D₃ reduced the promoter activity by 24.8% (Figure 4). An even greater reduction of BSP promoter activity by 1,25-dihydroxyvitamin D₃ was found in larger constructs that included further upstream sequences (J. J. Li and J. Sodek, unpublished work). These findings indicate that the binding of the 1,25-dihydroxyvitamin D₃ receptor protein may block transcription by interfering with TFIID binding to the inverted TATA element and that upstream sequences may be required either to stabilize the binding of the 1,25-dihydroxyvitamin D receptor protein or to provide sites for co-regulator(s) to bind. These possibilities are currently under investigation.

In summary, we have isolated a functional rat BSP gene promoter characterized by a putative inverted TATA box and an inverted CCAAT box, with a VDRE-like sequence overlapping the inverted TATA element. The existence of an inverted TATA element suggests that the orientation of a TATA box may not determine the direction of transcription, while the unique VDRE-like element indicates a novel mechanism for 1,25-dihydroxyvitamin D₃ to suppress gene transcription. Upstream elements, including a conserved homeobox-binding site and a novel 18 nucleotide palindrome, may be involved in the cell-specific expression of the BSP gene.

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