Cloning and functional characterization of the 5′-flanking region of the human bone morphogenetic protein-2 gene

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Bone morphogenetic protein-2 (BMP-2) is involved in bone formation, organogenesis or pattern formation during development. The expression of BMP-2 is regulated accurately and coordinately with that of other transforming growth factor-β (TGF-β) superfamily members. To elucidate the mechanism underlying the regulation of BMP-2 expression, a 6.7 kb *Spe*I–*Sal*I fragment, from the P1 phage library, encompassing the 5′-flanking region of the human BMP-2 gene, was isolated and sequenced. Transcription start sites were mapped by the 5′-rapid amplification of cDNA ends (RACE) method. It has been found that the human BMP-2 gene contains, largely, two promoter regions surrounded by GC-rich sequences with several Sp1 consensus motifs. The proximal promoter possesses a single start site, whereas several start sites are clustered in the distal promoter region. Neither TATA nor CAAT consensus sequences are found in the proximity of the start sites for either promoter. Interestingly, in no case is the transcription-initiation site common between the human and mouse BMP-2 genes, although the sequence of the BMP-2 gene is well conserved in the promoter region between two species. Transient transfection experiments with the reporter fused with various lengths of the BMP-2 promoter sequence demonstrated that there exist enhancer elements in an 1.1 kb GC-rich fragment covering both promoter regions. It is noteworthy that the enhancer elements are 5′-flanked by a 790 bp strong repressor element that is characterized by numerous AT stretches. This intriguing organization may be amenable to the tight control of the expression of BMP-2 that is essential for development or bone morphogenesis.

INTRODUCTION

Bone morphogenetic proteins (BMPs) were originally isolated from demineralized bone matrix to be used to induce bone formation when implanted into muscular sites [1]. After the cloning of their cDNAs, amino acid sequence analysis revealed that they are members of the transforming growth factor β (TGF-β) superfamily [2]. The bone-formation-inducing activity of BMPs clearly suggests that they are involved in skeletal development. Indeed, null mutation at the BMP-5 locus results in a short ear phenotype [3]. The phenotype of brachypodism in mice, characterized by skeletal abnormalities of their limbs and synovial joints, has been attributed to mutation in growth and differentiation factor-5 (GDF-5), another member of the BMP family [4]. Similarly, the frame-shift mutation of GDF-5 has been identified in patients with chondrodyplasia, a disorder that is phenotypically similar to murine brachypodism [5]. Furthermore, BMP-7 knockout mice show a defect in skeletal patterning in addition to eye and kidney defects [6].

BMP-2 is a representative member of the BMP family. BMP-2, as well as BMP-4, is implicated in the regulation of fracture repair [7]. Importantly, BMP-2 is likely to play a crucial role in embryogenesis, as evidenced from the lethal phenotype of mice lacking BMP-2 function [8]. Expression of BMP-2 mRNA in the apical ectodermal ridge suggests its role in limb patterning [9]. Other BMPs are also implicated in different stages of embryogenesis and organogenesis [9]. Various BMPs, including BMP-2, have been demonstrated to exhibit the same activity, such as the induction of ectopic bone formation *in vivo* and the promotion of osteogenesis and chondrogenesis of precursor cells *in vitro*, albeit to different degrees [10,11]. The overlapping biological activities of BMPs raise the possibility that each BMP molecule possesses distinct roles at determined time points in restricted sites during organogenesis or pattern formation, and it is therefore conceivable that the expression of the respective BMPs during development is regulated co-ordinately with that of other cognates of the BMP or TGF-β family in a temporally and spatially specific manner [12,13]. In fact, the expression pattern of BMP-2 is different from that of other BMP or TGF-β family members [14]. Therefore analysis of the mechanism underlying BMP-2 expression should give insights into the critical roles of BMP-2 in the developmental pathway.

We therefore cloned and sequenced the 5′-flanking region of the human BMP-2 gene. Transcription initiation sites were determined by the 5′-rapid amplification of cDNA ends (RACE) and the functional promoter activity of the 5′ upstream sequence was verified by transient transfection assays. These results proved that transcription of the BMP-2 gene was initiated at multiple sites surrounded by GC-rich sequences in which positive regulatory elements were located. Intriguingly, the enhancer elements were 5′-flanked by a strong repressor element characterized by a number of AT stretches. In the present study, these features of the human BMP-2 promoter region are discussed in comparison with those of the previously described mouse BMP-2 promoter [15].

MATERIALS AND METHODS

Cloning of human BMP-2 DNA 5′-flanking region

The primers B2-3: 5′-GTTTTGATGTCACCCCCGCTGTG-3′ (sense) and B2-7: 5′-CAGCTGGACTTAAGGCGTTTCCG-3′

Abbreviations used: BMP, bone morphogenetic protein; DMEM, Dulbecco's modified Eagle's medium; TGF-β, transforming growth factor-β; GDF-5, growth and differentiation factor-5; RACE, rapid amplification of cDNA ends; RT-PCR, reverse-transcribed PCR; SV40, simian virus 40.

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(antisense), corresponding to sequences in the coding region of the human BMP-2 gene, were selected for PCR screening of the P1 vector library [16] comprising human genome DNA. The conditions used for PCR were 35 cycles of 94 °C for 1 min, 60 °C for 30 s and 72 °C for 30 s. The screening of the P1 library by PCR was conducted as a customer service at Genome Systems Inc. (St. Louis, MO, U.S.A.), resulting in the isolation of two clones named P12621 and P12622. DNA was isolated from each vector with the standard alkaline lysis method [17]. The purified DNA was digested with various combinations of restriction enzymes, followed by 0.6% agarose gel electrophoresis. After the DNA fragments were transferred on to High Bond (Amersham, Bucks., U.K.) by Southern blotting [17], they were cross-linked by UV irradiation with Stratalinker 1800 (StrataGene, La Jolla, CA, U.S.A.). The digested DNA was hybridized with a digoxygenin-conjugated synthetic sense primer, 5′-GGGGACTTCTTGAA-CTTGCAGGGAGAATAACTTGCGCACCCCACTTTGC-GCCGGTGCCTT-3′, which corresponds to the 5′-untranslated region of the human BMP-2 cDNA [2]. Immunological detection of hybridized DNA was performed with a DIG detection kit II according to the supplier's manual (Boehringer Mannheim, Mannheim, Germany). As a result, it was found that a 6.7 kb *Spe*I–*Sal*I fragment contained the desired sequence. The fragment was cloned into the pBluescript SK(−) vector (Pharmacia Biotech, Uppsala, Sweden) that had been cut with a combination of the same restriction enzymes. The resultant clone was designated pBS6.7B2.

Sequencing of the 5′-flanking region of the human BMP-2 gene

The entire sequence of the cloned 6.7 kb *Spe*I–*Sal*I fragment spanning the 5′-flanking region of the human BMP-2 gene was determined with Cy5-labelled specific primers by the use of an ALF red autosequencer (Pharmacia Biotech). The sequencing was repeated at least three times in both directions. The fragments showing ambiguous sequences were subcloned into pUC18 (Pharmacia Biotech) and sequenced with the forward and reverse universal primers.

Cell culture

Three human osteosarcoma cell lines, Saos-2 [18], Hos [19] and MG63 [20], and mouse fibroblastic L cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (DMEM10) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Reverse-transcribed PCR (RT-PCR)

Poly(A)$^+$ RNA was prepared from the human osteosarcoma cell lines with the QuickPrep Micro mRNA purification kit (Pharmacia Biotech). cDNA was synthesized from the purified RNA by reverse-transcription using the kit from Amersham. Using the synthesized cDNA as template, PCR was performed with the above primers, B2-3 and B2-7, under the following conditions: 35 cycles of 94 °C for 1 min, 60 °C for 2 min and 72 °C for 2 min. The reaction was resolved by electrophoresis on a 2% agarose gel.

5′-RACE

The 5′-RACE reaction was performed with the Gibco BRL kit (Rockville, MD, U.S.A.). Total RNA was isolated from Saos-2 cells as previously described [21] and then subjected to reverse transcription with a gene-specific primer B2-11 (5′-TTAGG-AGACCGCAGTCCGTCTAAG-3′) corresponding to nucleotides +114 to +137 in Figure 2. After Glassmax purification and dC tailing, two consecutive PCR reactions were performed using the dC-tailed cDNA as template. In the first PCR reaction, the anchor primer (possessing an oligo-dG sequence at its 3′ end) and B2-9 (5′-CCGCAGTCCGTCTAAGAAGCA-3′; +109 to +129) were used under the following conditions: 35 cycles of 94 °C for 1 min, 65 °C for 2 min and 72 °C for 2 min. The second PCR used the abridged universal amplification primer and B2-12 (5′-GACACGTCCATTGAAAGAGCGT-3′; +81 to +102) as primers and the 100-fold diluted first PCR reaction as template. The reaction was allowed to proceed under the same conditions as those for the first reaction, except that a temperature of 70 °C rather than 65 °C was employed for the annealing step. After 2.5% agarose gel electrophoresis, three discrete bands appeared. The DNA fragments of each band were purified by GeneClean (BIO101, Vistam, CA, U.S.A.), cloned into pGEM T-Vector (Promega, Madison, WI, U.S.A.) and transformed into DH5α *Escherichia coli* cells. At least three independent colonies were picked up per PCR band. The purified plasmids were subjected to sequencing with the universal primers described above.

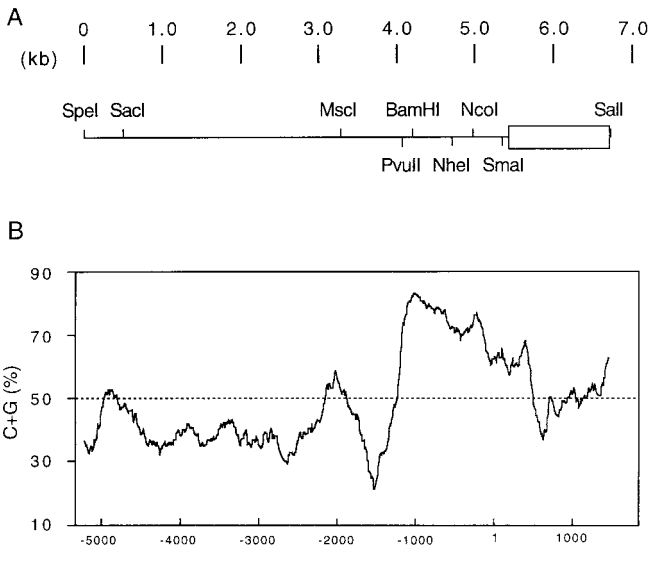

Figure 1 Structure of the human BMP-2 5′-flanking region

(**A**) Schematic representation of the restriction map for the insert of pBS6.7B2. An *Spe*I–*Sal*I fragment containing the 5′ upstream region of the human BMP-2 gene was cloned from the P1 bacteriophage library and sequenced entirely. The restriction sites used for subcloning or deletion mutant construction are indicated. The intron is represented by an open box. (**B**) GC content analysis of the sequence of the 6.7 kb *Spe*I–*Sal*I fragment per 200 bp segment (steps of 10 bp). The numbers are relative to the proximal start site, which is designated as nucleotide +1. The scale of the graph matches that of the restriction map in (**A**).

Construction of serially truncated promoter fusion plasmids

Plasmid ΔNc/pGL3 was constructed in the following three steps. A 370 bp *Nco*I–*Sma*I fragment (Figure 1) was first ligated into the *Nco*I–*Eco*RV sites of pGEM5zf(+) (Promega). The resulting vector was cleaved with *Not*I and *Apa*I and the excised fragment was cloned into *Not*I/*Apa*I cut pGEM11zf(+). Subsequent to digestion of the resultant plasmid with *Hin*dIII and *Sal*I, the released insert was isolated and fused to the pGL3 basic (pGL3B) vector (Promega) cut with the same restriction enzymes, generating the ΔNc/pGL3 construct. pGL3B contains luc+, a

mutant version of the firefly luciferase sequence with a stimulated activity, but lacks both eukaryotic promoter and enhancer. To generate ΔB/pGL3, ΔNc/pGL3 digested with *Bam*HI and *Nco*I was ligated with a 670 bp *Bam*HI–*Nco*I fragment released from pBS6.7B2. ΔNh/pGL3 was created by treating ΔB/pGL3 with *Nhe*I, followed by religation. ΔB/pGL3 was cut with *Sac*I and *Pst*I and ligated with an *Sac*I–*Pst*I fragment of pBS6.7B2. The constructed plasmid was termed B2/pGL3, the promoter–reporter construct carrying the longest BMP-2 5′-flanking sequence. B2/pGL3 in turn was used to obtain ΔP/pGL3 or ΔM/pGL3 by digestion with *Kpn*I and either *Pvu*II or *Msc*I, blunt-ending at the *Kpn*I site and self-ligation.

Construction of plasmids with a putative negative regulatory element in a heterologous construct

To join a potential repressor element to the simian virus 40 (SV40) promoter-driving luciferase gene, a 0.79 kb *Msc*I–*Pvu*II fragment was produced by PCR with ΔM/pGL3 as template. The primers used were 5′-ACGCGTCCACGTGTCACAGCCCTACTTC (sense) and 5′-AGATCTCTGGCGCGCCGGACCCTCGC (antisense). The restriction sites incorporated to facilitate the subsequent cloning are underlined. Each cycle consisted of 94 °C for 1 min, 60 °C for 2 min and 72 °C for 2 min for a total of 35 cycles. To ensure fidelity, a proof-reading DNA polymerase (LA *Taq* polymerase; Takara, Kyoto, Japan) was utilized for the reaction. The PCR products with the expected size were isolated after 1.5% agarose gel electrophoresis and cloned into a pGEM T-vector. The construct thus obtained was digested with *Mlu*I and *Bgl*II and the linearized fragment was religated into the pGL3 control vector (pGL3C, Promega) at the *Mlu*I and *Bgl*II sites, creating MP/pGL3C harbouring the negative regulatory element in the forward direction. To yield PM/pGL3C, the other derivative of pGL3C, which also contains the above repressor fragment, but in the reverse direction, MP/pGL3C was digested with *Bgl*II and blunt-ended. The digestion of the linearized plasmid with *Mlu*I liberated the fragment covering the putative repressor sequence. PM/pGL3C was created by fusing the isolated fragment to pGL3C at the blunt-ended *Sac*I and *Mlu*I sites. MP/pGL3C and PM/pGL3C were utilized to obtain the corresponding derivatives of the pGL3 promoter vector (pGL3P, Promega). MP/pGL3P was made by inserting the *Mlu*I–*Bgl*II fragment of MP/pGL3C into pGL3P via the *Mlu*I and *Bgl*II sites, whereas the ligation of the *Kpn*I–*Mlu*I fragment of PM/pGL3C with pGL3P cut with the same enzymes produced PM/pGL3P. The fidelity of the constructs was verified by restriction mapping. pGL3P contains the SV40 promoter sequence in front of the luc+ gene. The difference between pGL3P and pGL3C is only the additional presence of the SV40 enhancer in pGL3C.

Transient transfection experiments

Cells were plated in 24-well culture plates (Corning, Corning, NY, U.S.A.) with DMEM10 medium at a density of 4×10^4 per well. The next day, the cells were transiently transfected with 0.4 μg of the various promoter–reporter fusion plasmids in conjunction with the same amount of the pRLSV40 vector (Toyo Ink, Tokyo, Japan) by LipofectAMINE (Gibco BRL) according to the manufacturer's instructions. The pRLSV40 vector containing sea pangy (*Renilla*) luciferase driven by the SV40 promoter was included as an internal control. About 72 h after the transfection, the transfected cells were lysed and tested for the activity of two different luciferases by using the PicaGene dual assay (Toyo Ink). The chemiluminescence was measured by Luminoscan RS (Labsystems, Helsinki, Finland). The experiments were done in triplicate and repeated at least twice. Results are expressed as means ± S.E.M.

Analysis of the sequences

GC content analysis and comparison of the corresponding sequences of the BMP-2 gene between different species were carried out using Genetyx software (Software Development Co. Ltd., Tokyo, Japan). The transcription factor binding motif search was conducted with TFSEARCH (Akiyama, Y., http://www.rwcp.or.jp/papia/) based on the TRANSFAC database [22]. Matrix similarity scores were set at 90.0, except for an Sp1 consensus motif for which the threshold was lowered to 80.0.

Materials

Oligonucleotides were commercially available from Gleiner Japan (Tokyo, Japan) or Amersham Pharmacia Biotech Japan (Tokyo, Japan). Human genomic DNA was a product of Clontech. A ϕX174-*Hae*III digest (Takara) was used to provide DNA size markers. Restriction enzymes were obtained from New England Biolabs (Beverly, MA, U.S.A.), Toyobo (Osaka, Japan) or Takara. T4 DNA polymerase for blunt-end reactions was also purchased from Takara. *Taq* polymerase from Boehringer Mannheim was used for PCR unless otherwise stated. The ligation reaction was carried out with a DNA ligation kit obtained from Takara. Plasmids were purified on a Qiagen column (Qiagen, Hilden, Germany). DMEM was purchased from Gibco BRL.

RESULTS

Molecular cloning of the 5′-flanking region of the human BMP-2 gene

For the cloning of the 5′-flanking region of the human BMP-2 gene, we chose to employ the P1 bacteriophage library that can accommodate an insert of longer than 50 kb [16] to enhance the possibility of obtaining the entire portion of the desired sequence. Screening of the P1 phage library by PCR with a combination of primers specific for the human BMP-2 cDNA sequence gave rise to two clones. From one of the isolated phage clones, a 6.7 kb *Spe*I–*Sal*I fragment encompassing an immediate upstream sequence of the BMP-2 open reading frame, as identified by Southern blotting (results not shown), was subcloned into the pBluescript vector (Figure 1A). The sequence determined (Figure 2) could be aligned with the published cDNA sequence [2], substantiating the suspicion that the cloned gene was really derived from the human BMP-2 gene. The comparison further revealed that the genomic sequence diverged from the cDNA sequence just 8 bp upstream of the adenine of the initiation codon ATG, implying that there exists an approx. 1.2 kb intron immediately upstream of the first ATG. The intron–exon junctions conform to the GT/AG rule [23]. The mouse counterpart also has an intron with a smaller size (approx. 1.0 kb) at the same site [15], but the similarity of the whole intron sequence between the human and mouse genes was only 47%.

Identification of human BMP-2 transcription start sites

Human cells or tissues expressing BMP-2 were required to determine the transcription-initiation site of the gene; however,

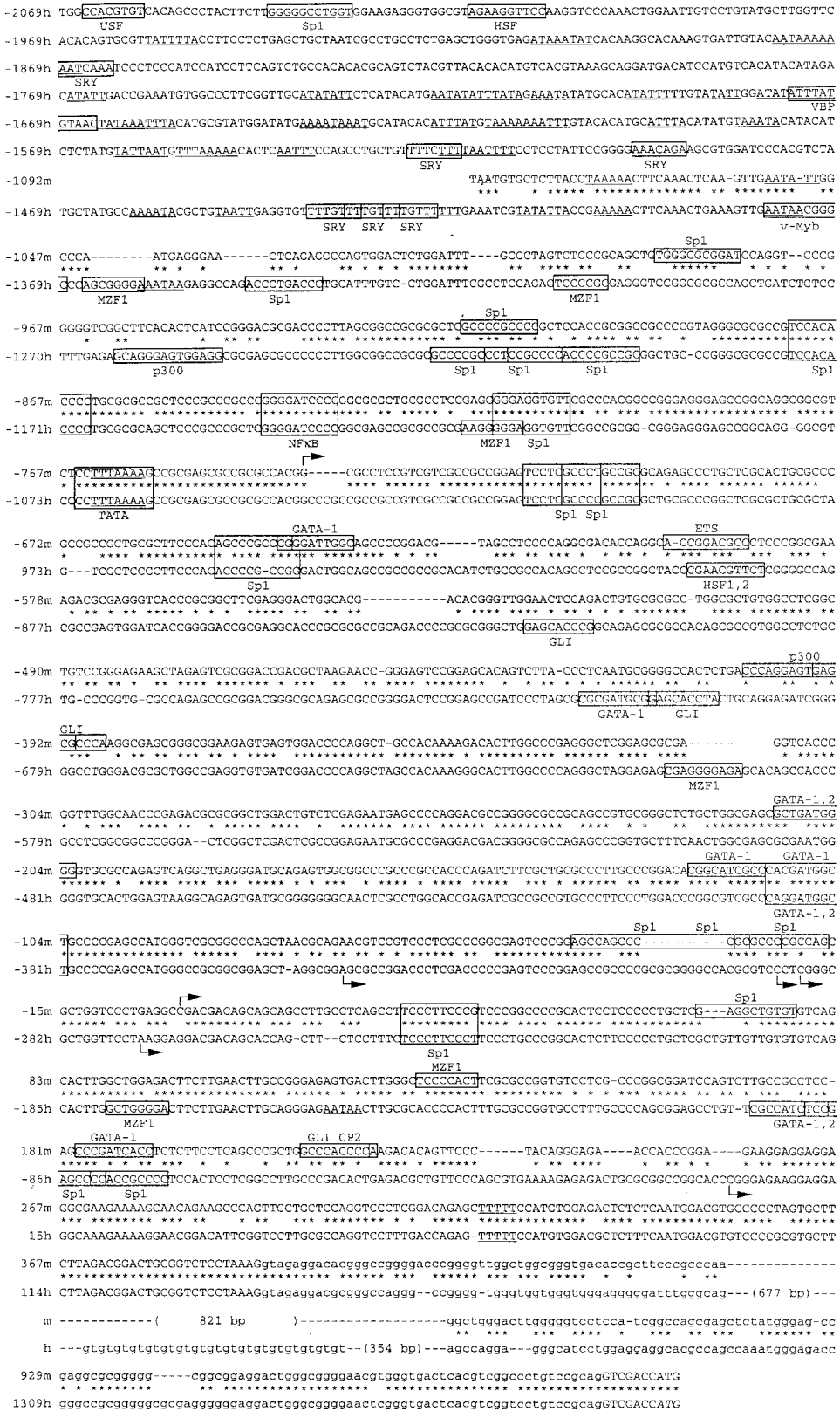

Figure 2 Alignment of the mouse (m) and human (h) nucleotide sequence of the BMP-2 gene 5′-flanking region

Numbers on the left refer to the position relative to the respective proximal transcription start sites (+1). The transcription start sites are indicated by arrows. AT tracts with more than five A/T repeats are underlined. Potential transcription factor binding motifs assigned by TFSEARCH are boxed. Also boxed are potential transcription factor binding sequences resembling the zinc finger protein GLI binding site (GACCACCCA) (these could not be searched by the TFSEARCH program). Introns are indicated by lowercase lettering. The GT repeat is absent in the intron of the mouse BMP gene. The 3′-end sequence CATG of the human BMP-2 gene derived from the literature is in italics.

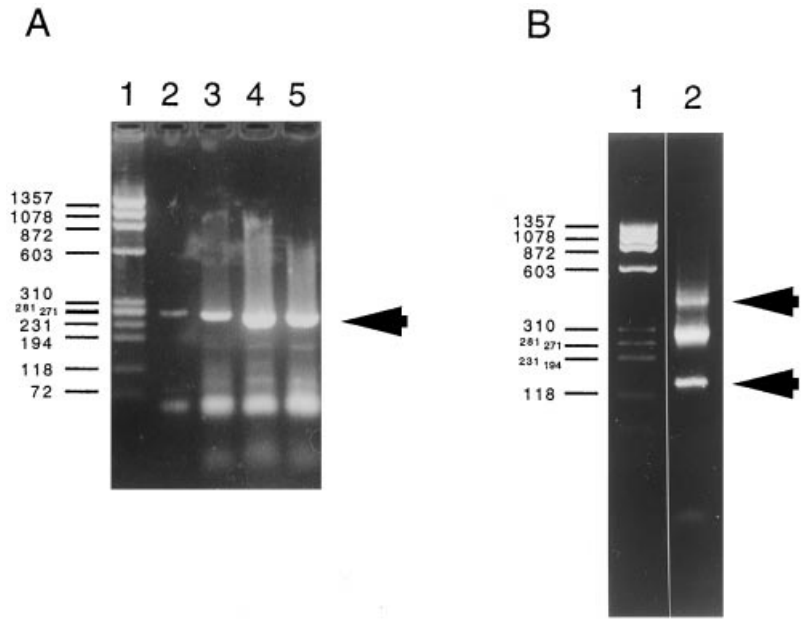

Figure 3 Analysis of the transcription start sites of BMP-2

(**A**) Poly(A)$^+$ RNA was prepared from human osteosarcoma cell lines and reverse-transcribed. The cDNAs generated from Hos (lane 3), MG63 (lane 4) or Saos-2 (lane 5) cells or human genomic DNA (lane 2) were used as templates for the specific PCR. The reaction was resolved by electrophoresis on a 2% agarose gel. DNA size markers are indicated in lane 1. The expected size of the PCR product specific for BMP-2 is 270 bp. An arrow indicates the specific band. (**B**) Total RNA obtained from Saos-2 cells was used for 5′-RACE analysis. The secondary PCR products were separated by 2.5% agarose gel electrophoresis (lane 2). Lane 1 contains DNA size markers. The bands related to the BMP-2 sequence are denoted by arrows.

the general difficulty in acquiring tissues or primary cultures of human origin forced us to resort to human osteoblastic cells as a substitute. The selected cell lines, Saos-2, MG63 and Hos were tested by RT-PCR for their expression of BMP-2. As indicated in Figure 3(A), all the cell lines examined definitely expressed BMP-2. The possibility of genomic DNA contamination was completely excluded by the fact that another PCR with intron-spanning specific primers for GDF-5 did not produce any band extended by the intron sequence when the same cDNA as prepared in this study was used as template (results not shown). From the RT-PCR analysis, one of the cell lines, Saos-2, was chosen as a source in the transcription start site study.

Total RNA obtained from the osteoblastic cells was used for the 5′-RACE analysis. After the first PCR no discrete band was observed by electrophoresis on a 2.5 % agarose gel. The dilution of the first PCR was subjected further to the nested PCR, resulting in three bands (Figure 3B). The respective PCR products were cloned from the bands that had different mobilities and sequenced for identification. The sequences of three independent clones isolated from the bottom band were identical, allowing for mapping of the start site to the last C in the sequence CCCGGG (-2 to $+4$ in Figure 2). On the other hand, the top band was, as expected from its broad nature, heterogeneous in terms of the possible transcription start site: two out of five clones indicated the same position (-272) as the start site and the remaining three clones mapped the start site to distinctive positions (-346, -290 and -287). The middle band turned out to be composed of a sequence unrelated to BMP-2. Thus, the human BMP-2 gene appears to possess largely two promoters; the proximal promoter contains a single predominant transcription start site whereas in the distal promoter the transcription begins at multiple sites.

Promoter activity of the human BMP-2 5′-flanking region

To investigate the cell-type-specific promoter activity of the human BMP-2 upstream region, a luciferase–reporter fusion plasmid with a 4.9 kb *Sac*I–*Sma*I fragment (Figure 1A) was constructed (B2/pGL3) and transiently introduced into Hos (osteoblastic) or L (fibroblastic) cells. Hos cells were employed as a representative osteoblastic cell line in this experiment because of their favourable transfection efficiency. When the construct was transfected into non-osteoblastic L cells, only a moderate promoter activity (a 2–3-fold increase over the promoterless pGL3B activity) was observed (Figure 4C), whereas an approx. 40-fold stimulation of the activity was induced by the same construct in Hos cells (Figure 4A). The cell-type-specific activity of the human BMP-2 promoter implicated by these results reflects either the presence of a specific positive regulator(s) or the absence of a repressor in the osteoblasts.

The 5′-flanking region of the human BMP-2 gene was delineated in more detail by carrying out transient transfection experiments with a series of nested deletion mutants using Hos cells as a host (Figure 4B). Removal of a *Sac*I–*Msc*I fragment (ΔM/pGL3) from B2/pGL3 completely removed the promoter activity in Hos cells; however, a more extensive deletion to the *Pvu*II site (ΔP/pGL3) restored the activity to about 70 % of the B2/pGL3 level. These findings imply that there exists a strong repressor element between the *Msc*I and *Pvu*II sites and at the same time a certain element within the *Sac*I–*Msc*I region functions as an anti-repressor. Deletion of a short *Pvu*II–*Bam*HI fragment (ΔB/pGL3) had a minimal effect on the transcriptional activity. Sequential deletion mutants (ΔNh/pGL3 and ΔNc/pGL3) to the *Nco*I site caused a gradual decrease in the promoter activity. The minimal construct (ΔNc/pGL3), consisting largely

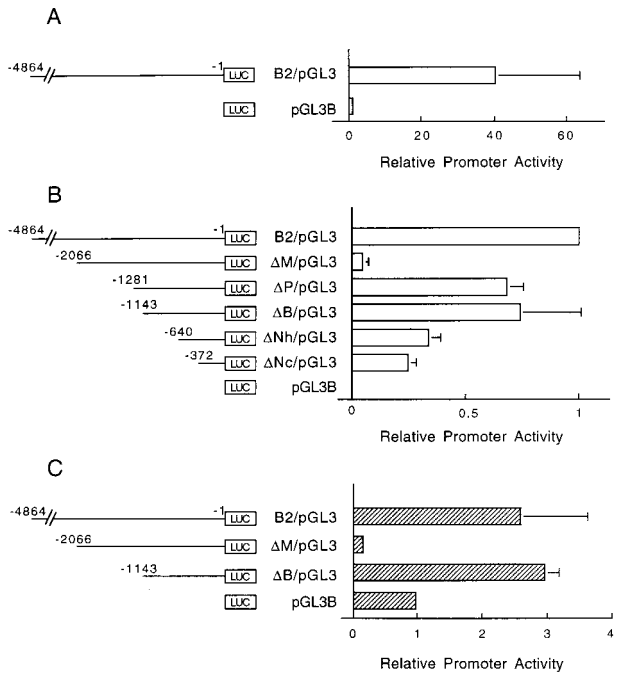

Figure 4 Transient expression of serial deletions of the human BMP-2 promoter

The activity of serially deleted promoters of human BMP-2 was assayed in osteoblastic Hos or non-osteoblastic L cells. Constructs are identified on the left of the diagram. The cells were transfected with the constructs along with pRLSV as an internal control. The cells were harvested 72 h later and tested for the different luciferase activities. The promoter activity, as expressed by the fire fry luciferase activity, was normalized to the sea pangy luciferase activity. The experiments were done in triplicate and repeated two or three times. The results are expressed as the mean ± S.E.M. (**A**) The promoter activity of B2/pGL3 in Hos cells. Results are indicated as a fold increase over the promoterless pGL3B vector. (**B**) The promoter activity of successive deletion mutants of the human BMP-2 promoter–reporter constructs in Hos cells. The activity of B2/pGL3 is set at 1, whereas the activity of pGL3B is set at 0. (**C**) Promoter activity of the successive deletion mutants in L cells. The normalized activity of the constructs is expressed as a fold stimulation over that of pGL3B.

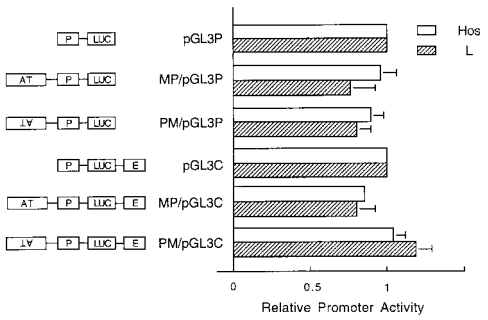

Figure 5 Transient expression of the putative repressor element–luciferase fusion plasmids constructed in a heterologous context

The putative repressor sequence was placed in front of the SV40 promoter in both directions with (pGL3/C) or without (pGL3/P) the SV40 enhancer elements. The resultant constructions were transfected into Hos or L cells, as described in the legend to Figure 4. The promoter activity is expressed relative to that of pGL3/C or pGL3/P in each cell line. The average promoter activity of pGL3/C, pGL3/P and pGL3B was 39.2, 7.0 and 0.42 respectively in Hos cells and 39.6, 40.1 and 1.5 respectively in L cells. Open bars indicate the activity in Hos cells and hatched bars that in L cells. The experiments were done in triplicate and repeated two or three times. The constructs are identified on the left of the diagram.

of the proximal promoter region, retained 25% of the promoter activity of B2/pGL3. In general, it is evident that the enhancer sequences contained in the 1.1 kb *Bam*HI–*Sma*I fragment contributed mostly to the activity of the human BMP-2 gene to drive transcription. Thus, the 5′ upstream region of the BMP-2 gene consists of positive regulatory elements bearing multiple transcription start sites and a 5′ negative regulatory element adjacent to the enhancer.

The presence of a strong repressor in the 5′-flanking region of the human BMP-2 gene raised the possibility that the lower promoter activity of B2/pGL3 in L cells might be ascribed to a greater suppressing effect exerted by the repressor element in the non-osteoblastic cells. If this is the case, the activity of the construct lacking the negative regulatory element would be derepressed in L cells, reaching the level of B2/pGL3 in Hos cells. To test this possibility, ΔM/pGL3 and ΔB/pGL3 were transfected into L cells. The results are depicted in Figure 4(C). ΔM/pGL3 displayed a significant reduction of the promoter activity in L cells, as in the case of Hos cells. Deletion of the repressor sequence (ΔB/pGL3) relieved the inhibition of the promoter activity but only to a limited extent, comparable with the original activity of B2/pGL3 in L cells. Hence, it can be concluded that a positive rather than negative factor would be responsible for the cell-type-specific promoter activity of B2/pGL3 in Hos cells.

Effects of a repressor element in a heterologous context

To determine whether the repressor sequence in the human BMP-2 gene could fulfill the requirements for a classical silencer [24], the promoter–reporter fusion plasmids were constructed in the context of a heterologous promoter and examined for luciferase-inducing activity. For this purpose, the *Msc*I–*Pvu*II fragment exhibiting repressor activity (Figure 4B) was placed, in both orientations, upstream of the pGL3C or pGL3P vectors in which the SV40 promoter drives the luc+ gene, with or without the SV40 enhancer respectively. The transient transfection studies illustrated in Figure 5 showed that the presence of the potential negative regulatory element did not cause a meaningful reduction in the promoter activity of pGL3P or pGL3C, regardless of the direction. Therefore, the *Msc*I–*Pvu*II repressor sequence in the human BMP-2 5′ upstream region cannot be described as a classical suppressor.

DISCUSSION

The 5′-flanking region of the human BMP-2 gene was cloned and sequenced to analyse how its temporal and spatial expression is achieved. The sequence determined in this study revealed that there is an intron of about 1.2 kb immediately upstream of the translational start codon in the human BMP-2 gene. Also, in the mouse counterpart, a 1.0 kb intron is present at the corresponding site [15]. The human BMP-2 5′ upstream intron is only 47% identical with the mouse intron; however, the sequences around the exon–intron boundaries at both 5′- and 3′-ends are well conserved for about 40 and 90 bp respectively (Figure 2). Interestingly, the dinucletotide $(GT)_{17}$ repeat that is associated with the Z-DNA configuration [25] is found in the 5′ upstream intron (+874 to +907 relative to the transcription start site) of the human BMP-2 gene. The dinucleotide tract is absent in the

intron of the mouse gene. The GT repeat is found in the 5′-flanking promoter region, not in the intron region, of the human GDF-5, another member of the BMP family, gene as well [26].

To locate the promoter region, the transcription-initiation sites were first determined by 5′-RACE using total RNA isolated from Saos-2 cells. In total, five positions were identified as transcription start sites, one of which is separated by more than 270 bp from the other clustered sites, indicating that the human BMP-2 gene contains, largely, two promoter regions. It should be noted that whereas a single position was defined as the start site for the proximal promoter, several start sites were used for the distal promoter. Likewise, two promoters are reported for the murine BMP-2 gene, but in this case both promoters use a unique initiation site (Figure 2). The similarity between the human and mouse BMP-2 genes in the promoter region 5′ upstream of the intron is higher than 75%, consistent with the previous report for the non-coding region sequences of the human and mouse T-cell receptor genes [27]; however, in no case is the start site of the BMP-2 genes identical in the two species (Figure 2). In the human gene both the proximal and distal promoters lack a canonical TATA box or a consensus initiator element; both are buried in the GC-rich region with several weak and strong Sp1 consensus motifs. In contrast, the distal initiation site of the mouse BMP-2 gene is preceded by a TATA-like sequence while the proximal promoter region contains potential Sp1 binding sites. Since it has been established that Sp1 functions to govern transcription start site utilization by binding to the consensus sequence in a GC-rich region [28,29], it is likely that the different locations of Sp1 binding sites in the GC-rich regions account for the different mechanisms of the transcription-initiation site selection of the BMP-2 gene between the two species. The species-specific difference in the transcriptional start regulation is also observed for the human and mouse P-selectin genes [30].

The transient transfection experiments showed that there exist positive regulatory elements in a 1.1 kb *Bam*HI–*Sma*I fragment and also a negative regulatory element in a 0.79 kb *Msc*I–*Pvu*II region in the human BMP-2 5′ upstream region. Potential transcription factor binding sites sought by TFSEARCH [22] are indicated in Figure 2, with consensus motifs for the zinc finger protein, GLI [31], which is a mediator of 'hedgehog', a secreted protein with the ability to induce BMP-2 [32]. The repressor sequence found between the *Msc*I and *Pvu*II sites abrogated the activity of the BMP-2 promoter almost completely in the transient transfection experiments, suggesting that initiation-complex formation was disrupted by some factor(s) binding to the sequence. However, the inhibitory sequence cannot be considered as a classical silencer because the *Msc*I–*Pvu*II fragment was unable to suppress the activity when placed in front of the heterologous SV40 promoter in either direction, irrespective of the existence of the additional enhancer element (Figure 5). In striking contrast to the downstream GC-rich enhancer region, the *Msc*I–*Pvu*II region is highly AT-rich (Figure 1B) and inspection of the sequence exhibited numerous AT stretches (underlined in Figure 2). It is tempting to speculate that a strong inhibition of the BMP-2 promoter activity was exerted through the binding of a nuclear protein binding to the AT-rich sequences in the repressor element. One candidate is HMG-I(Y) [33,34], which binds to the AT tracts separated by appropriate sizes and is able either to activate [35,36] or inhibit [37,38] transcription by creating bending in the DNA configuration, depending on the different promoter contexts. This organization of the BMP-2 promoter region, in which the AT-rich strong suppressing element is adjacent to the GC-rich enhancer elements in the 5′ direction, may be amenable to the complicated regulations necessary for the accurate expression pattern of BMP-2 during development or bone morphogenesis. Since the promoter activity was recovered by the addition of the upstream *Sac*I–*Msc*I fragment, an anti-repressor or enhancer element in the sequence is capable of antagonizing the repressor element.

Finally, the potency of the B2/pGL3 promoter activity was much greater when transfected into Hos osteoblastic cells than into L fibroblasts. The osteoblast-specific activity of the BMP-2 promoter is in good agreement with the previous findings that BMP-2 is most abundantly present in bone, despite its prevalent expression in a variety of tissues [39,40]. In this study, it was also confirmed by RT-PCR that BMP-2 was prominently expressed by all the human osteoblastic cells examined (Figure 3A). However, because deletion of the repressor sequence did not enhance the promoter activity to the predicted level, the diminished activity of the BMP-2 promoter–luciferase fusion plasmid in the fibroblastic cells does not appear to result from the presumed negative regulatory factor specifically expressed in the non-osteoblastic cells. Rather, it is reasonable to propose cell-type-specific positive regulation of BMP-2 expression in the osteoblastic cells.

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