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Genomic Organization of the Human Bone Morphogenetic Protein-4 Gene: Molecular Basis for Multiple Transcripts

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The structure of the human bone morphogenetic protein-4 (BMP-4) gene has been characterized from a genomic cosmid clone of about 38 kb. The transcriptional unit of the human BMP-4 gene is encoded by 5 exons and spans approximately 7 kb. The exon/intron organization of the human BMP-4 gene is similar to that of the mouse gene, with notable sequence differences in the 5' non-coding exons. The human BMP-4 gene has at least two functional promoters, which are used in a cell type specific manner. This observation is of fundamental relevance for understanding the specific role of BMP-4 in skeletal development and bone remodeling. © 1996

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Bone morphogenetic protein-4 (BMP-4) is a member of the BMP family, which belongs to the transforming growth factor- β (TGF- β) superfamily (1). BMPs can induce *de novo* cartilage and bone formation, and appear to be essential for skeletal development during mammalian embryogenesis. Subcutaneous or intramuscular implantation of recombinant BMP-4 induces the formation of new bone. Furthermore, BMPs are involved in promoting bone healing in mammals (2). Recently, it has become evident that during fracture healing the concentration of BMP-4 increases dramatically (3, 4). These observations suggest an important role of BMP-4 in bone remodeling and fracture repair.

BMP-4 is not only involved in osteoinductive events, but also in many other developmental processes such as tooth morphogenesis (5), hematopoiesis (6) and neuronal differentiation (7). BMP-4 is one of the best evolutionary conserved growth factors and is able to induce mesoderm formation. This was first described for BMP-4 homologues in *Drosophila melanogaster* (8) and *Xenopus laevis* (9), but also in mammals BMP-4 seems to be an important factor in mesoderm formation and patterning (6, 10).

Although the role of BMP-4 seems to be crucial during many stages of development, little is known about its transcriptional regulation. Two human BMP-4 transcripts have been described, which are completely identical in their coding region, but are different in their 5' non-coding regions. One of these transcripts was isolated from an osteosarcoma cell line (1), the other was isolated from a prostate cancer cell line (11). The regulatory mechanisms underlying specific BMP-4 expression in human are unknown, but may reflect a combination of transcriptional and post-transcriptional control. For understanding the molecular mechanisms of the transcriptional control of human BMP-4, it is essential to isolate and characterize the complete BMP-4 gene. Although the overall structure of the mouse BMP-4 gene has already been described (12, 13), very little is known about the structure and functional organization of the human BMP-4 gene. A first report about the genomic structure of the human BMP-4 gene describes a fragment with two exons, containing the complete coding region (14). However, non-coding exons and promoter regions have not been identified yet.

In this report, we describe the isolation and characterization of a cosmid clone containing the

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entire human BMP-4 gene. Furthermore, a specific RT-PCR was developed and used to detect BMP-4 specific transcripts in several human cell lines. The results presented here suggest that the gene contains at least two functional promoters.

MATERIALS AND METHODS

Screening of a human genomic library. A 1.3 kb mouse full length BMP-4 cDNA fragment (mmBMP4) was obtained by PCR on a lambda gt 10 8½ day C57BL mouse cDNA library (gift from Dr. B. L. M. Hogan, Vanderbilt University, Nashville, TN), using primers F4 (5' CGCGGATCCCAAGT TTGTTCAAGATTGGCT 3') and R4 (5' CGCGGATCCGCCTGATCTCAGCGGCACCC 3'), based on human BMP-4 cDNA sequence HSBMP2B (14). This probe was used to screen a human genomic cosmid library (16; gift from Mr. J. van Groningen, University of Nijmegen, The Netherlands). Approximately 3×10^5 cosmid clones were screened under non-stringent conditions in $5\times$ SSC, $5\times$ Denhardt's, 0.5% SDS, 100 μ g/ml denatured herring sperm DNA at 65 °C overnight with the 32 P-labeled mmBMP4 probe, and filters were washed up to $0.1 \times$ SSC/0.1% SDS at 65 °C prior to autoradiography. One of the positive clones was subsequently purified to homogeneity by tertiary screening using the mmBMP4 probe.

Genomic clone mapping and DNA sequencing analysis. Cosmid DNA was purified and submitted to Southern blot analysis after digesting with appropriate restriction enzymes to generate a physical map. The DNA was transferred onto Hybond-N membrane (Amersham) and probed with the mmBMP4 fragment or BMP-4-specific primers (F4 and R4) to determine the orientation of the gene and approximate position of exons. Fragments that hybridized with these probes were subcloned into pBluescript II KS(-) (Stratagene) and analysed by DNA sequence analysis using the T7-Sequencing Kit (Pharmacia). For identification of the splice junctions of the BMP-4 gene, internal primers were chosen near possible splice junctions. These primers were used for direct cosmid DNA sequencing, according to the dsDNA Cycle Sequencing System (GibcoBRL). Genomic sequences were compared with the mouse BMP-4 gene (17) and several human BMP-4 cDNA sequences using the Fasta computer analysis program (Caos/Camm, The Netherlands).

Messenger RNA extraction and RT-PCR. Several human cell lines were used, including human foreskin fibroblast (HF-V32), human embryonic kidney (HEK-293), primary human skin fibroblast (F11), undifferentiated Tera-2 cells (Tera2EC), RA-differentiated human Tera-2 cells (Tera2RA) (18) and the osteosarcoma cell lines MG-63, U-2 OS and Saos-2 (originally obtained from ATCC). A 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with 10 % fetal calf serum (FCS) was used for culturing F11, HEK-293, HF-V32, MG-63, Saos-2 and U-2 OS; α MEM/10 % FCS was used for Tera2EC and Tera2RA (5). Messenger RNA was extracted using the Micro-Fast Track mRNA isolation kit, according to the supplied protocol (Invitrogen Corp., San Diego, CA). From each sample approximately 20 ng mRNA was reverse transcribed according to the manufacturer's protocol using 100 pmol of oligo(dT) primer and 200 units SuperScript-II (Gibco-BRL). Thereafter, 10% of the reaction volume were amplified in a PCR reaction, using 25 pmol of each primer (sense and antisense) and 2.5 U *Taq* DNA polymerase (Gibco-BRL). PCR reactions were transferred directly from ice to 95 °C for 5 min., followed by 36 cycles at 95 °C for 1 min., 52 °C for 1 min., 72 °C for 2 min. and subsequently an extra extension step at 72 °C for 7 min. The nested PCR was under the same conditions, only with an annealing temperature of 54 °C instead of 52 °C. The sequences of the oligonucleotide primers for RT-PCR were the following: F1A (5' ATCCGAGCTGAGGGACGCGAGCCT 3'), R1C (5' CGTGTCACATTGTGGTG-GACC 3'), F1B (5' TGGCGAGCCCGCTACTGCAGGGAC 3'), R1D (5' GCATTCGGTTACCAGGAATCATGG 3'), F2A (5' AGCGCAGGCCGAAAGCTGTTC 3'), F2B (5' CGTTTTCTCGACTCCGGGAAC 3'). The integrity of the amplified fragments was confirmed by Southern blot hybridization, using a 32 P-labeled exon 3 specific-primer (F4).

RESULTS

Isolation of the Human BMP-4 Gene

A 1.3 kb mmBMP4 probe was used to screen a human genomic cosmid library. This mouse cDNA probe was used because of the high degree of sequence conservation of BMP-4 cDNAs in different species (11). One of the positive cosmid clones with a 38 kb insert was further purified and analyzed. Restriction fragments, obtained by several enzymatic digestions of the isolated cosmid DNA, were analyzed by Southern blot hybridization with BMP-4 specific probes. Positive fragments were subcloned and their nucleotide sequences were determined. The obtained sequences were analyzed by nucleic acid homology searches, showing that the 38 kb genomic clone contained the entire BMP-4 gene. In order to determine the exon/intron structure, we isolated and sequenced two human BMP-4 transcripts from Tera2RA cells (results not shown). Sequence analysis revealed that one transcript was identical to a previously described cDNA from an osteosarcoma cell line (19) and that the other transcript was identical to a cDNA from a prostate cancer cell line (11). Given the sequence information of both transcripts, together with the above men-

tioned partial genomic sequences and genomic Southern blot analysis, we were able to resolve the exon/intron structure. We found that the gene consists of 5 exons, spanning approximately 7 kb (fig. 1). In addition, comparison of both transcripts with the genomic structure indicated that one transcript contains exon 1, 3, 4 and 5 (named BMP4.1) and that the other contains exon 2, 3, 4 and 5 (named BMP4.2). Both transcripts and the nucleotide position of the introns are shown in Figure 2. To further characterize the human BMP-4 gene, we partially sequenced the exon/intron boundaries, using a combination of synthetic primers near possible splice sites (fig. 2) and subcloning of exon/intron containing fragments. The exact localization and sequence of splice sites is shown in Figure 3. All of the exon/intron boundaries defining the splice sites are conform the consensus sequence of AG at the 3' acceptor splice site and GT at the 5' donor splice site.

BMP-4 mRNA Expression

A specific RT-PCR method was used to detect the presence of the closely related BMP-4 mRNAs in different tumor cell lines. Cell lines derived from various tissues were chosen on basis of previous reports on BMP-4 expression (1, 14). The BMP4.1 transcript is indicated by PCR using the primers F1A and R1C, followed by a nested PCR with the primers F1B and R1D. To indicate the BMP4.2 transcript, a PCR using the primers F2A and R1C was performed, followed by a nested PCR with the primers F2B and R1D. After amplification with the nested primer combinations, fidelity of the BMP-4-specific PCR products was confirmed by hybridization using primer F4, an exon 3 specific primer. In fig. 4, a Southern blot is shown, containing the specific RT-PCR products indicating the presence of the two BMP-4 transcripts in various cell lines. Both transcripts were detected in the osteosarcoma cell lines U-2 OS and Saos-2. This was also the case for the embryonal kidney (HEK-293), the foreskin fibroblast (HF-V32) and the Tera-2RA cells. However, in Tera-2EC cells, the skin fibroblast (F11) and the osteosarcoma cell line MG63, only the BMP4.2 transcript was present.

DISCUSSION

The genomic organization of the entire human BMP-4 gene has been characterized from a 38 kb cosmid clone. BMP-4 is encoded by a single gene, which was previously mapped to chromosome 14q22-23 (15). The transcriptional unit of the gene contains 5 exons and spans approximately 7 kb. Sequence comparison with the mouse BMP-4 gene shows a high degree of homology in the coding regions and a similar exon/intron structure. However, notable sequence differences are observed in the non-coding exons 1 and 2. The human exon 2 shows only slight homology with a sequence in the mouse BMP-4 gene, starting about 150 bp upstream of the mouse exon 2 (12).

Molecular analysis showed that from the human BMP-4 gene at least two mRNAs are transcribed, which share the same translation product but differ in their 5' region. The low level of expression, the high homology and the small difference in size between both BMP-4 transcripts, make it difficult to detect both transcripts by Northern blot hybridization. Therefore, we developed a sensitive RT-PCR to investigate the difference in BMP-4 transcription in various tumor cell lines.

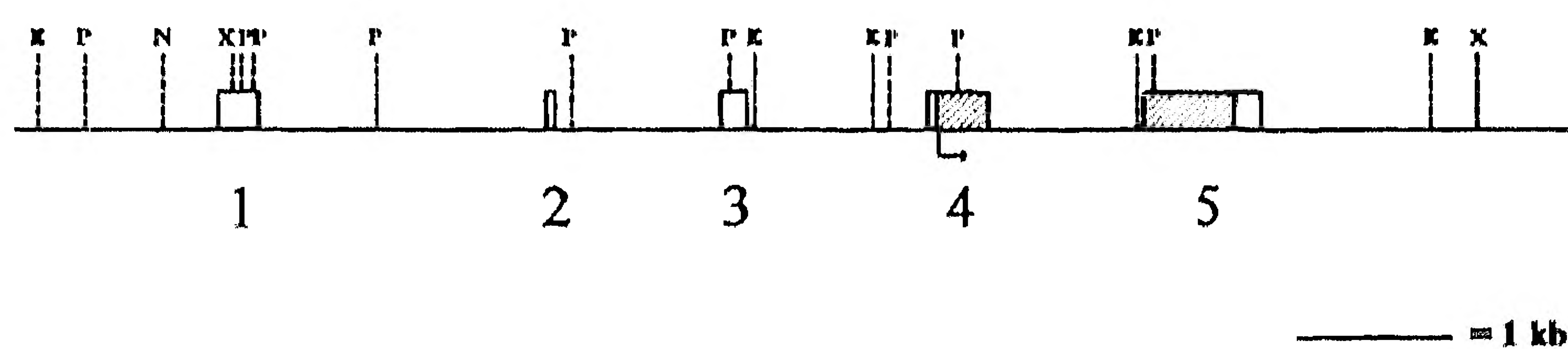


FIG. 1. Structure and restriction map of the human BMP-4 gene. About 10 kb of the gene containing the five exons is shown. Exons (solid bars) are indicated by the numbers 1 to 5. The coding region (shaded box) is shown together with the location of the proposed translation start site within exon 4 (arrow). Only restriction sites relevant to the present work are indicated: E, *EcoRI*; X, *XhoI*; P, *PstI* and N, *NcoI*.

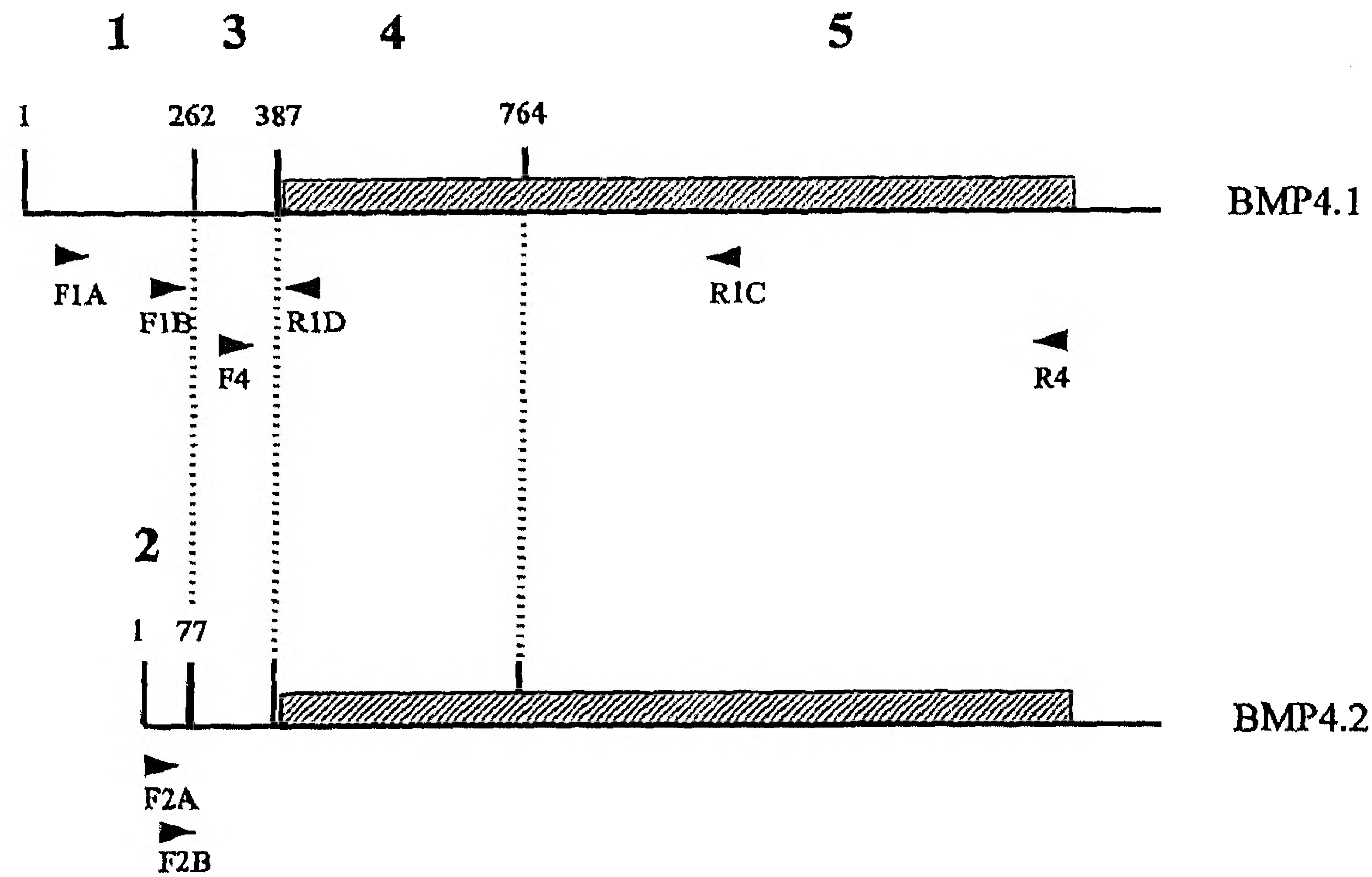


FIG. 2. Location of primers for the two BMP-4 transcripts. The location of introns (vertical lines), together with the first nucleotide position of each exon (numbers), are indicated. The coding region (shaded box) and exons (bold numbers) are shown. The first BMP-4 transcript (BMP4.1), is the same as HSBMP2B (19), which contains exons 1, 3, 4 and 5. From the second transcript (BMP4.2) only the length of exon 2 is indicated (11), which is spliced to exons 3, 4 and 5 at nucleotide position 262 of the HSBMP2B sequence (19). The sequences of exons 3, 4 and 5 of both transcripts are exactly the same. Primers (arrow heads) are shown as reverse (R) and forward (F).

We found that both transcripts are expressed in a cell type depending manner. Interestingly, the BMP-4.2 transcript was detectable in all cell lines tested, while the BMP4.1 transcript was not.

Both transcripts, together with the exon/intron sequences, suggest the presence of at least two putative promoters. A differential regulation of these promoters might explain the difference in cell line-specific expression of both mRNAs. Most likely, one promoter is located upstream of the non-coding exon 1 and the other is present just upstream of the non-coding exon 2. Because of the genomic organization and the RT-PCRs, it is unlikely that BMP4.2 is a splicing variant of BMP4.1. Upstream of the coding region (exon 4 and 5), a non-coding region (exon 3) is present in both transcripts. Since the translation start site is located in exon 4, the transcription initiation from both promoters will result in identical protein products.

A variant of the BMP-4 mRNA has recently been cloned from a human placental cDNA library (20). The 5' non-coding region of this transcript is different from the previously described mRNAs

EXON	(SIZE)	Exon 3'	intron (size)	Exon 5'
Exon 1	(> 262 bp)	CCTATGgtgagcaaggctacc.....	intron 1 (1.9 kb).....	_____
Exon 2	(> 77 bp)	AACATGgtlgggatttccttc.....	intron 2 (1.1 kb).....	aaatattccttttagGAGCCA
Exon 3	(125 bp)	CTGTCAgtcagtagacacctg.....	intron3 (1.2 kb).....	ctcccctcccagAGACAC
Exon 4	(377 bp)	ACGAAGgtcagtccttaaac.....	intron4 (1.0 kb).....	cctaactgtgcctagAACATC
Exon 5	(>987 bp)	_____		

FIG. 3. Exon-intron boundaries of the BMP-4 gene. Nucleotide sequence for each intron-exon boundary and size of each exon and intron are shown. The 5' site of exon 1 and 2 is not shown, because of the unknown transcription initiation site. The 3' site of exon 5 is not shown because of its unknown poly(A+) addition site.

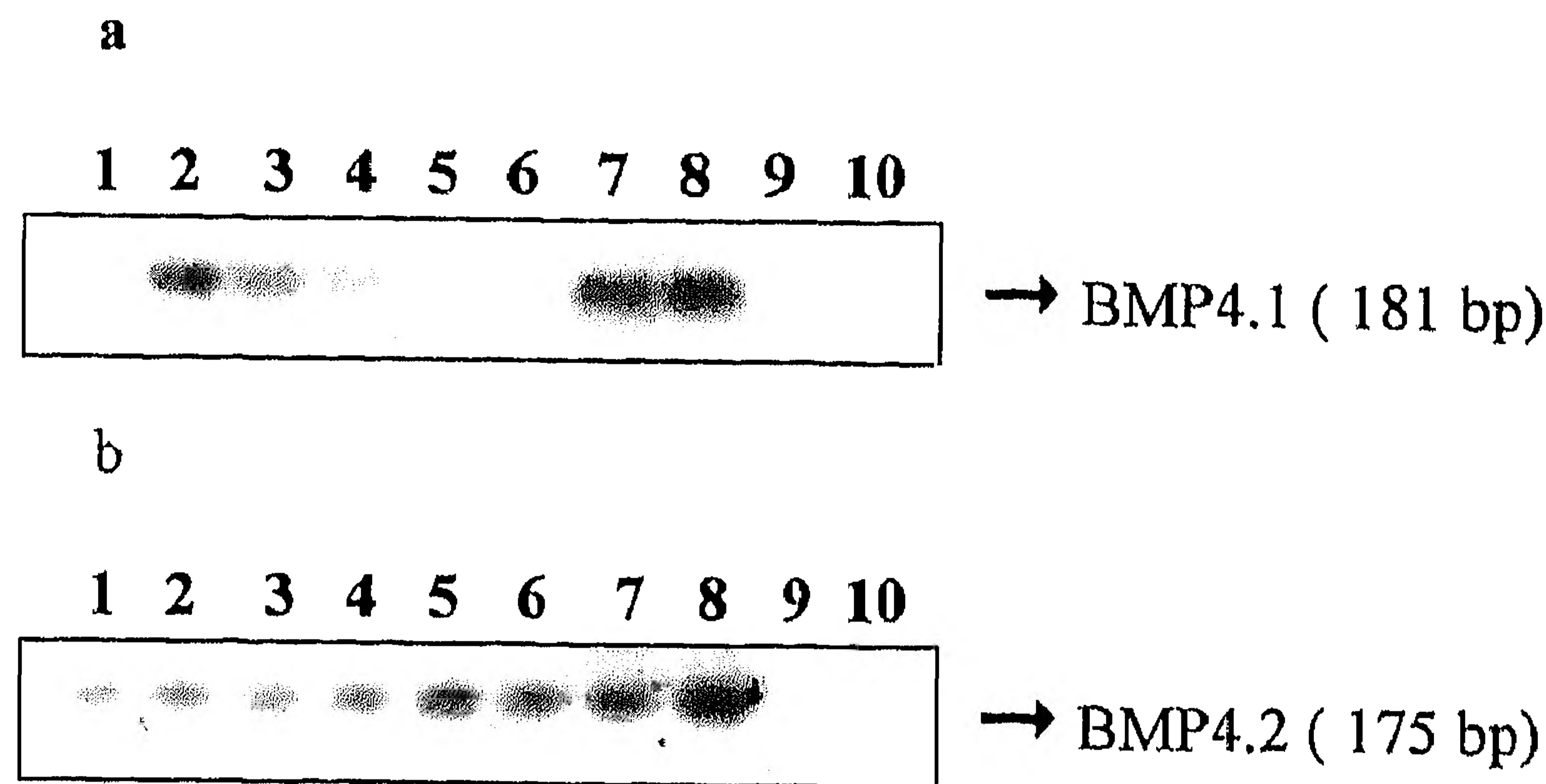


FIG. 4. Southern blot of the BMP-4 specific RT-PCR products, derived from several human cell lines. (a) BMP4.1-specific PCR fragment of 181 bp. (b) BMP4.2-specific PCR fragment of 175 bp. The PCR fragments were hybridized with a ^{32}P -labeled exon 3 probe, which is primer F4 in figure 3. Human cell lines and negative controls are numbered, 1: MG-63, 2: U-2 OS, 3: HF-V32, 4: HEK-293, 5: F11, 6: EC, 7: RA, 8: Saos-2, 9: RT- H_2O and 10: H_2O .

and the deduced amino acid sequence indicated deletion of six N-terminal amino acids. Therefore, it can not be excluded that additional promoters might be present, upstream of the two putative promoters reported here. Various transcripts have also been observed for a BMP-4 homologue in *Drosophila melanogaster*, decapentaplegic protein (dpp) (21). These multiple dpp-specific mRNAs have been shown to result from several promoters and alternatively spliced 5'-untranslated exons. In the mouse, the BMP-4 gene also contains multiple non-coding regions and alternative promoters, which is in agreement with our results for the human gene (12, 13).

Regulation of BMP-4 gene expression and further evaluation of the different BMP-4 mRNAs will shed new light on the detailed role of this gene during osteogenesis. The presence of the two separate promoters and the diverse transcripts in several cell lines derived from different tissues, suggests a cell or tissue specific regulation of BMP-4 gene expression. To gain more insight in the transcriptional regulation of the BMP-4 gene in man, further characterization of the two putative promoters is in progress.

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