Identification of a Novel Bone Morphogenetic Protein-responsive Gene That May Function as a Noncoding RNA*

(Received for publication, December 16, 1997, and in revised form, April 23, 1998)

Kohsuke Takeda‡, Hidenori Ichijo‡§§, Makiko Fujii†, Yoshiyuki Mochida‡§, Masao Saitoh‡§, Hideki Nishitoh‡§, T. Kuber Sampath∥, and Kohei Miyazono‡

From the §Department of Biochemistry, The Cancer Institute, Tokyo, and ¶Department of Biomaterials Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8455, Japan. Tel.: 81-3-5803-5471; Fax: 81-3-5803-0192; E-mail: ichijo.det2@dent.tmd.ac.jp.

Bone morphogenetic proteins (BMPs)/osteogenic proteins (OPs), members of the transforming growth factor-β superfamily, have a wide variety of effects on many cell types including osteoblasts and chondroblasts, and play critical roles in embryonic development. BMPs transduce their effects through binding to two different types of serine/threonine kinase receptors, type I and type II. Signaling by these receptors is mediated by the recently identified Smad proteins. Despite the rapid progress in understanding of the signaling mechanism downstream of BMP receptors, the target genes of BMPs are poorly understood in mammals. Here we identified a novel gene, termed BMP/OP-responsive gene (BORG), in C2C12 mouse myoblast cell line which trans-differentiates into osteoblastic cells in response to BMPs. Expression of BORG was dramatically induced in C2C12 cells by the treatment with BMP-2 or OP-1 within 2 h and peaked at 12–24 h, whereas transforming growth factor-β had a minimal effect. BMP-dependent expression of BORG was also detected in other cell types which are known to respond to BMPs, suggesting that BORG is a common target gene of BMPs. Cloning and sequence analysis of BORG cDNA and the genomic clones revealed that, unexpectedly, the transcript of BORG lacks any extensive open reading frames and contains a cluster of multiple interspersed repetitive sequences in its middle part. The unusual structural features suggested that BORG may function as a noncoding RNA, although it is spliced and polyadenylated as authentic protein-coding mRNAs. Together with the observation that transfection of antisense oligonucleotides of BORG partially inhibited BMP-induced differentiation in C2C12 cells, it is possible that a new class of RNA molecules may have certain roles in the differentiation process induced by BMPs.

Bone morphogenetic proteins (BMPs) are osteogenic proteins (OPs), members of the transforming growth factor-β (TGF-β) superfamily, were originally identified by their activity to induce bone formation in vivo (1, 2). The BMP family includes various proteins, which can be divided into several subgroups based on their structural similarity; i.e. a group containing Drosophila decapentaplegic gene product, BMP-2, and BMP-4, a group containing Drosophila 60A gene product, OP-1/β-MP-7, OP-2/BMP-8, BMP-5, and BMP-6/Vg1, a group containing growth/differentiation factor-5, -6, and -7, and other members (3–5).

In vitro studies have revealed that BMPs have various biological effects on osteoblasts and chondroblasts, e.g. stimulation of proteoglycan synthesis in chondroblasts, and induction of collagen, alkaline phosphatase, and osteocalcin during chondrogenic and osteogenic differentiation (6–8). BMPs appear to exert various effects on many other cell types and play critical roles in embryonic development. For instance, null mutation in BMP-2 gene leads to defects in amnion/chorion and cardiac development (9), and OP-1-deficient mice die shortly after birth because of poor kidney development and have eye defects and skeletal abnormalities (10, 11).

BMPs transduce their signals through binding to two different types of serine/threonine kinase receptors, type I and type II (12). Upon ligand binding followed by the formation of heteromeric receptor complexes, type I receptors are phosphorylated by type II receptors, and subsequent activation of the catalytic activity of type I receptor kinase is essential for signaling (13–16). Signaling by these receptors is mediated by the recently identified Smad proteins (17, 18). In the case of BMPs, phosphorylation of Smad1 (19–23) by the activated type I receptors allows association of Smad1 with Smad4 (24), and the complex moves into the nucleus, wherein Smads regulate the transcription of a subset of target genes.

In Xenopus embryos, Smad5 is also able to mediate BMP signaling (25). In activin signaling, Smad2 interacts with the activin-response element of Mix.2, an immediate early activin-response gene, in concert with FAST-1, a novel member of the winged-helix family of putative transcription factor (26), whereas DNA-binding partners of Smad1 or Smad5 have yet been unknown in BMP signaling. Certain Smads have been shown to directly bind to DNAs (27, 28).

In order to elucidate how the Smad proteins and other transcription factors function in mediating BMP signals, and whether the signaling pathways not using the Smad proteins

osteogenic protein; BORG, BMP/OP-responsive gene; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde phosphate dehydrogenase; RACE, rapid amplification of cDNA ends; SINE, short interspersed nucleotide element; TGF-β, transforming growth factor-β; bp, base pair(s); ORF, open reading frame; PCR, polymerase chain reaction; RT, reverse transcriptase.

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are also involved in BMP signaling, it is necessary to identify and analyze the genes directly induced by BMPs. A number of target genes of decapentaplegic gene product, the *Drosophila* counterpart of BMP-2, have been reported (18, 29). In *Xenopus*, homeobox-containing genes, *Mix.1* (30), *Xvent-1* (31, 32), *Xvent-2* (33–35), and *msx1* (36), and erythroid transcription factors, *GATA-1* (37) and *GATA-2* (38), have been shown to have immediate early response to BMPs. The mammalian counterparts of these genes may function as direct target genes of BMPs; however, little is known to date in mammals about the BMP-responsive genes except for TGF-β-inducible early gene (TIEG), a putative zinc finger protein which is induced by BMP-2 as well as TGF-β (39).

In the present study, we report the isolation of a novel gene, termed BMP/OP-responsive gene (BORG), of which expression was regulated by either BMP-2 or OP-1 in BMP-responsive cells. Interestingly, it lacks any extensive open reading frames (ORFs) and contains a cluster of multiple interspersed repetitive sequences in its middle part. A possibility that BORG may function as a noncoding RNA in the BMP-induced differentiation process is discussed.

**MATERIALS AND METHODS**

**Cell Culture**—Mouse muscle myoblast C2C12 cells (40) and mouse embryo fibroblast C3H10T1/2 clone 8 were obtained from the American Type Culture Collection. ST2 mouse bone marrow stromal cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). C2C12 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Nissui) containing 15% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin). When the C2C12 cells were treated with BMP-2, OP-1, or TGF-β, medium was replaced by DMEM containing 5% FBS and antibiotics. C3H10T1/2 cells and ST2 cells were maintained in basal medium Eagle’s with Earle’s salts (Life Technologies, Inc.) and antibiotics.

**Total RNA Isolation**—Total RNA was isolated from the cells by using Isogen (Wako), and poly(A)+ RNA was purified as binding to Oligotex-dT<sub>30</sub> Super (Takara Biomedicals) as described by the manufacturer’s instructions.

**Differential Display**—C2C12 cells were cultured in DMEM containing 10% FBS, to reach confluency; the serum was reduced to 5%, and the cells were subjected to digestion with DNase I (MessageClean kit, GenHunter) for 2 hours at 37 °C overnight with 5 × SSPE (1 × SSPE is 180 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>/H<sub>2</sub>O, 1 mM EDTA), 50% formamide, 0.5% Denhardt’s solution, 0.5% SDS, 20 μg/ml salmon sperm DNA. The filters were washed twice in 2 × SSPE, 0.1% SDS at 43 °C for 15 min, once in 1 × SSPE, 0.1% SDS at 43 °C for 30 min, and once in 0.1 × SSPE, 0.1% SDS at room temperature for 15 minutes, followed by the films being analyzed using a Fuji BAS 2000 Bio-Imaging Analyzer (Fuji Photo Film).

**RT-PCR**—One μg of total RNA was reverse transcribed into single strand cDNA using Superscript Preamplification System (Life Technologies, Inc.) as described by the manufacturer’s instructions. PCR was performed in a 50-μl reaction containing 1 × PCR reaction buffer (Boehringer Mannheim), 200 μM dNTPs, 0.2 μM B-S4 primer (5'-TAATTGGAAGACCCTAGTACTAGG-3'), 0.2 μM B-A54 primer (5'-TCCTGTTGAAAGAAGCTGCC-3'), 1 μl of single strand cDNA solution, and 2.5 units of Taq polymerase (Boehringer Mannheim). PCR conditions were 94 °C (5 min), 25 cycles of 94 °C (30 s), 55 °C (30 s), and 72 °C (30 s), followed by 10 min at 72 °C. A second round of PCR was performed with 2 μl of the first reaction as a template in the same reaction mixture except for the use of the internal primers, B-S5 (5'-CACGGGGTTTTTTTTTTTTTTTTTTTTTTTGTATTGATGA-3') and B-ASX1 (5'-CAGCTCGAGCTGACCATGATGTCGTTCC-3') instead of B-S4 and B-A54. The second PCR conditions were 94 °C (1 min), 15 cycles of 94 °C (30 s), 55 °C (30 s), and 72 °C (30 s), followed by 10 min at 72 °C. Specificity of the PCR products was confirmed by digestion with EcoRI and EcoRV. Primers for the mouse or rat GAPDH (CLONTECH) were also used as loading controls for the RT-PCR procedure.

**5'-RACE**—Rapid amplification of cDNA ends (5'-RACE) was performed using a Marathon cDNA Amplification Kit (CLONTECH). Using 1 μg of poly(A)+ RNA isolated from OP-1-treated C2C12 cells, a library of adapter-ligated double strand cDNA was constructed as described by the manufacturer’s instructions. For the initial attempt to obtain a full-length cDNA of BORG, two sequential antisense primers, B-AS1 (5'-ATGACGGTGGGAGCCTGTTACGTAC-3') and B-AS2 (5'-CCAAGTGGGGCTCACTGTTGATGGC-3'), were designed from the sequence of the cDNA fragment obtained by the differential display. To isolate the 5'-end of BORG cDNA, B-AS6 (5'-AGCCTGCAGGTGGATT- TAAAC-3') and B-AS3 (5'-GT GGTAAGCTGATCTGGTGGATGACCT-3') were designed from the sequence of the 5'-region of the differentially displayed cDNA library clone P69 (see below). PCR reaction was performed in a 50-μl reaction containing 50 μg Tri斯-HCl, pH 9.2, 14 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.75 mM MgCl<sub>2</sub>, 200 μM dNTPs, 0.2 μM B-A51 primer, 0.2 μM adapter primer 1 (AP1, CLONTECH), 0.5 μl of adapter-ligated double strand cDNA solution, 2.5 units of Taq/Two DNA polymerase mixture (Expand Long Template PCR system, Boehringer Mannheim), and 0.5 μg of TaqStart Antibody (CLONTECH). PCR conditions were 94 °C (15 min), followed by 20 cycles of 94 °C (30 s), 55 °C (30 s), and 72 °C (30 s) for 1 min. A second round of PCR was performed with 0.5 μl of the first reaction as a template in the same reaction mixture except for the use of B-AS2 primer and nested adapter primer 2 (AP2, CLONTECH) instead of B-AS1 and AP1. The second PCR conditions were 94 °C (1 min), followed by 20 cycles of 94 °C (30 s) and 65 °C (4 min). The PCR product was subcloned into pGEM-T vector (Promega), sequenced, and used as a probe for cDNA library screening.

**Preparation of cDNA Library and Isolation of cDNA Clones**—Using poly(A)+ RNA isolated from OP-1-treated C2C12 cells, an oligo(dT)-primed cDNA library with 1 × 10<sup>9</sup> independent clones was prepared by Uni-ZAP XR Gigapack II Gold Cloning kit (Stratagene). The amplified cDNA library was plated and lifted onto nylon filters (Hybond-N, Amersham), and immobilized by UV cross-linking. The duplicate filters were probed with the 32P-labeled 5'-RACE product at 65 °C overnight in the hybridization buffer containing 5 × SSPE, 5 × Denhardt’s solution, 0.5% SDS, 20 μg/ml salmon sperm DNA. The filters were washed at 65 °C twice in 2 × SSPE, 0.1% SDS for 15 min, once in 1 × SSPE, 0.1% SDS for 30 min, and once in 0.1 × SSPE, 0.1% SDS for 15 min, followed by autoradiography. The positive clones were isolated and rescued into phagemid SK+ (Stratagene). Nucleotide sequencing was performed on both strands.

**Isolation of BORG Genomic Clones**—One million clones of a 129SV<sup>msx1</sup> mouse genomic library (Stratagene) were screened with the full-length BORG cDNA as a probe. DNA fragments isolated from the positive clones and subjected to digestion with appropriate restriction enzymes to generate a physical map. The digested DNA was

**Northern Blot Analysis**—Two μg of poly(A)+ RNA was denatured, separated on a 1.2% agarose-formaldehyde gel, and blotted onto a nylon filter (Hybond-N, Amersham) with 20 × SSC (1 × SSC is 15 mM sodium citrate, 150 mM NaCl). Subcloned cDNA fragments from differential display and cDNAs for rat osteocalcin and glyceraldehydephosphate dehydrogenase (GAPDH) (gifts of Dr. S. Oida) were labeled with 32P by a Ready-To-Go DNA Labeling Kit (Pharmacia). Hybridization was performed at 43 °C overnight in 5 × SSPE (1 × SSPE is 180 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>/H<sub>2</sub>O, 1 mM EDTA), 50% formamide, 0.5% SDS, 20 μg/ml salmon sperm DNA. The filters were washed twice in 2 × SSPE, 0.1% SDS at 43 °C for 15 min, once in 1 × SSPE, 0.1% SDS at 43 °C for 30 min, and once in 0.1 × SSPE, 0.1% SDS at room temperature for 15 minutes, followed by the films being analyzed using a Fuji BAS 2000 Bio-Imaging Analyzer (Fuji Photo Film).
also probed with various portions of BORG cDNA to determine their location in the genome of BORG. Fragments that hybridized with the probes were subcloned into pBluescript SK(+) for nucleotide sequencing.

Antisense Oligonucleotides—Antisense oligonucleotides at nucleotide positions from 902 to 921 of BORG cDNA (Fig. 5) were designed to hybridize to BORG RNA. Nucleotide sequences are: antisense, 5'-CGAGGCCCACTACTCAAGTT-3'; sense, 5'-AAGTGGATATGGCATCTGG-3'. Both were synthesized as phosphorothionate oligonucleotides and high pressure liquid chromatography-purified by Greiner Japan (42). For transfection, 5 μM of each oligonucleotide was mixed with 2 μl/ml Tfx-50 (Promega) in DMEM containing 5% FBS, and added to C2C12 cells in the presence or absence of 300 ng/ml BMP-2. Total RNA extraction followed by RT-PCR for detecting the expression of BORG was done 6 h after the transfection as described above. Alkaline phosphatase activity was measured 36 h after the transfection as described previously (43).

RESULTS

Identification of a Novel Target Gene of BMPs—To identify a novel target gene of BMPs, we first examined the OP-1 responsiveness in a mouse myoblast cell line C2C12, which was reported to trans-differentiate into osteoblastic cells in response to BMP-2 (44). C2C12 cells were found to start to express osteocalcin mRNA (see below), as well as alkaline phosphatase activity (data not shown), representative markers for osteoblastic phenotype, by 24 h after the treatment with 300 ng/ml OP-1. In contrast, C2C12 cells not treated with OP-1 did not undergo such osteoblastic changes (data not shown), suggesting that C2C12 cells provide a useful system for the differential screening of OP-1-induced gene expression. We applied an mRNA differential display method (41) by using poly(A)+ RNA obtained from OP-1-treated or -untreated C2C12 cells. The C2C12 cells were maintained in DMEM containing 15% FBS. When the cells reached confluency, the serum was reduced, and high pressure liquid chromatography-purified by Greiner Japan (42). For transfection, 5 μM of each oligonucleotide was mixed with 2 μl/ml Tfx-50 (Promega) in DMEM containing 5% FBS in the presence (+) or absence (−) of 300 ng/ml OP-1 for the indicated time. Poly(A)+ RNA (2 μg) extracted from the cells was subjected to Northern blot analysis using BORG PCR fragment clone DD-10 as a probe. The filter of OP-1-treated cells was rehybridized with the rat osteocalcin cDNA probe. The amount of mRNAs was verified by rehybridizing the filters with GAPDH probe.

Fig. 1. Representative differential displays (A) and Northern blot analysis (B) of BORG. A, differential display was carried out using poly(A)+ RNA isolated from C2C12 cells untreated (−) or treated (+) with 300 ng/ml OP-1 for 2 h. 35S-Labeled PCR products were separated on a 6% denaturing polyacrylamide gel. Representative PCR products identified by using two different combinations of the primers are shown. The positive bands are marked by arrows. The differential display using the same set of the primers was performed three times with similar results. B, the differentially expressed product, clone DD-10, was labeled with 32P and used for Northern blot analysis of RNA from C2C12 cells untreated (−) or treated (+) with 300 ng/ml OP-1 for 2 h. The amount of mRNAs was verified by rehybridizing the filter with a GAPDH probe.

Fig. 2. Time course expression of BORG and osteocalcin mRNA in C2C12 cells. Confluent C2C12 cells were incubated with DMEM containing 5% FBS in the presence (+) or absence (−) of 300 ng/ml OP-1 for the indicated time. Poly(A)+ RNA (2 μg) extracted from the cells was subjected to Northern blot analysis using BORG PCR fragment clone DD-10 as a probe. The filter of OP-1-treated cells was rehybridized with the rat osteocalcin cDNA probe. The amount of mRNAs was verified by rehybridizing the filters with GAPDH probe.
BORG RNA in C2C12 cells. Confluent C2C12 cells were incubated with DMEM containing 5% FBS in the absence (control) or presence of time. Poly(A) was calculated as relative activity against the background signal and graphical representation of the results. The amount of each transcript was quantitated on a Fuji BAS2000 Bio-Imaging Analyzer and plotted to give a graphical representation of the results. The amount of each transcript was calculated as relative activity against the background signal and normalized by RNA loading on the gels. Relative changes were calculated by standardizing the 0-h time point as a basal level.

respond to BMPs. In ST2 mouse bone marrow stromal cells (45) and C3H10T1/2 mouse embryo fibroblast (46), BORG was found to be induced within 1 h after the treatment with BMP-2 (Fig. 4), suggesting that BORG is a common target gene of BMPs in BMP-responsive cells.

cDNA Cloning of BORG—To obtain a full-length cDNA for BORG, we applied 5'-RACE to poly(A)+ RNA isolated from OP-1-treated C2C12 cells by using two nested antisense primers designed from the sequence of the original PCR clone, DD-10. Specifically amplified products were subcloned into plasmid vectors, and two independent clones were sequenced. These clones encoded overlapping PCR products of 2,528 and 2,455 bp long, but a few nucleotides of these clones were different from each other in the overlapping region probably due to misincorporation of deoxynucleotides during the PCR procedure (data not shown).

Next, a cDNA library was constructed using poly(A)+ RNA isolated from OP-1-treated C2C12 cells and probed with the longer 5'-RACE product (2,528 bp). Several overlapping clones were obtained, and a clone, termed P69, yielded a 2,455-bp nucleotide sequence with a polyadenylation signal, AATAAA, followed by a poly(A) tail. The majority of the other clones were different from each other in the overlapping region probably due to misincorporation of deoxynucleotides during the PCR procedure (data not shown).

To determine the 5'-end sequence of BORG RNA, we again applied 5'-RACE using two sequential antisense primers designed in the 5'-region of P69 and the cDNA templates used in the initial 5'-RACE. Sequencing of the specifically amplified products yielded an additional six nucleotides at the 5'-end of the cDNA. Thus, the combined nucleotide sequence of the 5'-RACE product and P69 was a putative full-length cDNA for BORG, containing a polyadenylation signal, AATAAA, at the 3'-end (nucleotide 2,812) (Fig. 5).

When a fragment (nucleotides 911–1,273) of BORG cDNA was used in Northern blot analysis as a probe, we detected OP-1-induced expression of the same sized transcript as that observed by using the original PCR clone, DD-10, as a probe (data not shown). These results indicate that the cDNA clone obtained from the OP-1-treated C2C12 cDNA library corresponded to a transcript of BORG.

Sequence Analysis of BORG—A search of sequence data bases using the BLAST program (47) revealed an unexpected feature of BORG. Although no cDNA or mRNA sequence homologous to BORG transcript was detected in the data bases, mouse genomic sequence of origin region repeat-1a transposon-like element, clone origin region repeat-P (GenBank accession number: MMU17092) was highly homologous (76.7% identical) to a part of BORG cDNA (nucleotides 607–1,041), which indicates that BORG transcript contains an interspersed repetitive sequence (Fig. 5). To further investigate whether other interspersed repetitive sequences were involved in BORG cDNA sequence, we used RepeatMasker program2 that screens DNA sequences for interspersed repeats known to exist in mammalian genomes. BORG cDNA contained a cluster of homologous regions to four types of interspersed repetitive sequences in tandem; sequence of nucleotides 354–535 was homologous to type B4A of the short interspersed nucleotide element (SINE) (62.6% identical) (48), sequence of nucleotides 536–704 was homologous to the long terminal repeat region of origin region repeat-1B (70.2% identical), a member of a superfamily of mammalian apparent long terminal repeat-retrotransposons (49), sequence of nucleotides 709–1,042 was homologous to the internal sequence of origin region repeat-1A (78.7% identical), another member of mammalian apparent long terminal repeat-retrotransposons, and sequence of nucleotides 1,043–1,343 was homologous to RMER4 (62.6% identical), a long terminal repeat sequence that was not fully characterized in the RepeatMasker program or previous publications. These repetitive elements are frequently found within introns but much less in exons. Even if found in exons, they are usually found within 5'- or 3'-noncoding exons (50).

Another unexpected feature of BORG was the lack of any extensive ORFs in the cDNA sequence because of the high density of stop codons in all three reading frames (Fig. 6). The longest ATG-initiated ORF was found in nucleotides 911–1,273

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1 Smit, A. F. A., and Green, P., RepeatMasker, http://ftp.genome.washington.edu/RM/RepeatMasker.html.
which contained 363-bp long nucleotides with a favorable context for initiation of translation (51) with a purine at the $2^{\text{nd}}$ position (AGTATG T). However, this ORF (911–1,273) was preceded by 13 ATG codons, two of which occurred in strong contexts for initiation (nucleotides 670–676, GTCATG G and nucleotides 739–745, GTGATG G). Therefore, translation efficiency of the ORF (911–1,273) should be significantly reduced as reported by Kozak (51). In addition, the ORF (911–1,273) was completely included in the cluster of multiple interspersed repetitive sequences, and had no homology to any known coding sequences. Thus, the ORF (911–1,273) was unlikely to encode a protein. Four other ORFs longer than 100 bp were detected; two were in reading frame 1 (nucleotides 1, 111 to 1,344 and 1,687 to 1,905) and the other two were in reading frame 3 (nucleotides 1,602 to 1,739 and 2,412 to 2,543). However, none of them occurred in strong context for initiation. Taken together with the fact that these four ORFs were preceded by long 5′-noncoding regions including multiple ATG codons, these ORFs were also unlikely to function as coding sequences.

To examine whether the BORG transcript encodes a protein, in vitro transcription and translation of the full-length cDNA for BORG was performed by using rabbit reticulocyte lysates. We could not detect any specific protein products from BORG cDNA (data not shown). Although it cannot be completely excluded that BORG may encode a small peptide, these results together with the unusual structural features of BORG cDNA strongly suggest that BORG transcript may act as a noncoding RNA.

Genomic Organization of BORG—To negate the possibility that BORG is a processed pseudogene, we determined the genomic structure of BORG (Fig. 7). Using BORG cDNA as a probe, we isolated several overlapping genomic clones from a mouse genomic library. Detailed restriction mapping and Southern blot analyses of the clones and sequencing of their subclones revealed that BORG consisted of three exons interrupted by two introns. The sequences of the exon-intron bound-

FIG. 6. ORF analysis of BORG cDNA. The relative positions of all ATG codons and termination codons (TGA, TAA, and TAG) are indicated by open triangles and vertical lines, respectively, for each of the three reading frames.

DISCUSSION

In the present study, we identified a novel gene, termed BORG, in a C2C12 mouse myoblast cell line by using an mRNA...
We demonstrated that expression of BORG was strongly induced by either BMP-2 or OP-1 in C2C12 cells, whereas TGF-β, another member of TGF-β superfamily, had a minimal effect. The induction of BORG was first detected 2–3 h after the treatment with BMP-2 or OP-1 and peaked after 12–24 h by Northern blot analysis in C2C12 cells (Figs. 1B, 2 and 3). Although the kinetics of BORG induction by BMP-2 were essentially identical to that by OP-1, BMP-2 had a slightly stronger activity in BORG induction as compared with OP-1, which correlated well with alkaline phosphatase and osteocalcin inducing activity by BMP-2 and OP-1 (data not shown). In contrast, TGF-β, which does not induce an osteoblastic phenotype in C2C12 cells, induced BORG expression very weakly (Fig. 3). Moreover, the kinetics of BORG induction by TGF-β, which peaked after 3 h and rapidly decreased after this, was clearly different from that by BMP-2 or OP-1. Supposing that BORG is a specific target gene for OP-1 and BMP-2 but not TGF-β, BORG might also be sensitive to certain nonspecific stimulations such as TGF-β, but only specific stimulations for osteoblastic differentiation such as BMP-2 and OP-1 may strongly increase and maintain its expression level for a longer period. BMP-2-dependent expression of BORG was also detected in two other cell types, ST2 and C3H10T1/2, both of which are known to respond to BMPs (45, 46), suggesting that BORG is a common target gene of BMPs.

In Xenopus, homeobox-containing genes, Mix.1 (30), Xvent-1 (31, 32), Xvent-2 (33–35), and msx1 (36), and erythroid transcription factors, GATA-1 (37) and GATA-2 (38), have been shown to be immediate early response genes of BMPs. The counterparts of these genes are likely candidates for immediate early response genes of BMPs in mammalian cells. Because of the rapid responsiveness of BORG to BMPs together with the fact that cycloheximide failed to inhibit the induction of BORG by OP-1 (data not shown), BORG may be classified as an immediate early response gene of BMPs. No such genes have been reported in mammalian cells to date, except for a putative zinc finger protein, called TGF-β-inducible early gene (TIEG), which is induced by BMP-2 as well as TGF-β (39). Therefore, detailed analysis of the regulatory mechanism of BORG expression will allow us to elucidate the precise molecular pathways involved in BMP signaling.

Sequencing analysis of BORG cDNA revealed several unexpected features of BORG cDNA. One of them is the existence of a cluster of multiple interspersed repetitive sequences in the middle part of the cDNA (Fig. 5). A large fraction of the mammalian genome is composed of interspersed repetitive sequences. Most numerous such sequences are the short and long interspersed nucleotide elements represented in the human genome by Alu and L1 sequences, respectively. Mammalian apparent long terminal repeat-retrotransposons form a class of repetitive elements distinct from SINEs and long interspersed nucleotide elements (49). The cluster of multiple interspersed repetitive sequences of BORG cDNA includes one region related to SINE and three regions related to mammalian apparent long terminal repeat-retrotransposons. The significance of the cluster of multiple interspersed repetitive sequences in BORG is unknown. The lack of any extensive ORFs is another feature of BORG. Because of the high density of stop codons in all three reading frames (Fig. 6), even the longest ATG-initiated ORF in BORG cDNA contained only 363 bp with poor contexts for coding a peptide. CTG or ACG also serves naturally as a start codon (51); however, the longest ORF was still the 399-bp CTG-initiated ORF started at nucleotide 875 with the upstream strong ATG codons, again suggesting that it is unlikely to encode a long peptide. These unusual structural features of the cDNA suggest that BORG encodes a noncoding RNA.

Recently, considerable attention has been attracted to two mammalian genes, H19 (52–54) and Xist (55–57), both of which
function as untranslated RNAs. The structural features of the two genes resemble that of BORG in that they are spliced, polyadenylated, but have no extensive ORFs. H19 is implicated in imprinting of the insulin-2 and insulin-like growth factor 2 gene (53), and is demonstrated to have tumor-suppressor activity (54). Xist RNA acts in the nucleus and is essential for inactivation of most genes along the X chromosome in female (57). Thus, the characterization of these two RNA molecules supported a notion that untranslated RNAs can play important biological roles. In addition, although less characterized than H19 and Xist, three other noncoding RNAs have been reported to date. His-1, cloned from a common retroviral insertion site in murine leukemia virus-induced myeloid leukemia (58), and bice, cloned from a common retroviral insertion site in avian leukosis virus-induced B-cell lymphoma (59), may also encode noncoding RNAs, both implicated in growth control and oncogenesis. A novel synapse-associated noncoding RNA, TH4, has been cloned as a candidate synaptic regulatory molecule from rat (60). The function of TH4 in synaptic nuclei remains unknown, but interestingly, TH4 cDNA has a short region containing homologous sequence to B1 SINE in the middle part of the cDNA, which is a structural feature in common with BORG. Therefore, BORG might be a new member of a growing family of Xist-type and that transfection of antisense oligonucleotides of a functional protein product, may be important in order to disclose the functional roles of BORG in BMP-induced alkaline phosphatase activity, further experiments will be needed to disclose the functional roles of BORG in BMP-induced osteoblast differentiation.

Acknowledgments—We are grateful to M. Asashima, S. Takahashi, H. Okabayashi, and S. Noji for valuable discussion. We thank S. Oda for cDNAs for osteocalcin and GAPDH.

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