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Regulation of Osteoblast-Specific Factor-1 (OSF-1) mRNA Expression by Dual Promoters as Revealed by RT-PCR

Masahiro Sato,*[,1] Kenji Miyado,* Makoto Okazaki,† and Minoru Kimura*[,‡]
**Molecular Medicine Research Center, The Institute of Medical Sciences and ‡Department of Molecular Life Science, Tokai University, Bohseidai, Isehara, Kanagawa 259-11, Japan; †Laboratory for Bone Research, Discovery Research Laboratories, Research and Development Division, Nippon Hoechst Marion Roussel Limited, 1-3-2 Minamidai, Kawagoe, Saitama 350-11, Japan*

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OSF-1 (osteoblast-specific factor-1), which is also referred to as p18, HBBM, HB-GAM, HBGF-8, HARP, HBNF, and pleiotrophin, is a 121-amino acid polypeptide that can induce neurite outgrowth *in vitro* and is highly expressed in several tissues during fetal development but exhibits expression restricted to brain and bone tissues in adults. We have reported the genomic structure of mouse OSF-1 gene, in which the open reading frame spans four exons and at least two additional 5′-UTR exons (upstream exon U2 and downstream exon U1) exist. From analysis of isolated cDNAs, two types of cDNAs were identified: one has a sequence for U1 and U2 and the other has a sequence for an intron (present between U1 and U2) and U1. This suggests that the OSF-1 gene utilizes two alternative promoters, a distal and a proximal promoter, designated promoters II and I, respectively, for the translation initiation site (ATG). Promoter II is thought to exist upstream of the intron, while promoter I is present in the intron. RT-PCR was employed to examine which OSF-1 promoters are used during development and in various cell lines. In adult mice (aged 2 months), usage of promoter I was predominant, and OSF-1 mRNAs were expressed in many organs including brain and bone. At one fetal stage (E-19), promoter I was active in the major organs including brain, liver, kidney, and intestine, while promoter II was active only in the brain. In the cell lines examined, usage of promoter I was frequent, while promoter II was active only in a few cell lines such as MC3T3-E1 (cultured for 7 days) and C3H10T1/2. These findings suggest that OSF-1 may play fundamental roles in differentiation, growth and maintenance of adult organs as well as in embryogenesis, and indicate that the expression of OSF-1 is regulated, at least in part, by the usage of different promoters in the mouse. © 1997 Academic Press

Osteoblast-specific factor-1 (OSF-1) (1) [also known as HARP (2), retinoic acid-induced heparin binding protein (RIHB) (3,4), pleiotrophin (PTN) (5), heparin-binding growth-associated molecule (HB-GAM) (6), p18 (7,8), heparin-binding growth factor-8 (HBGF-8) (9), heparin-binding brain mitogen (HBBM) (10) and heparin-binding neurotrophic factor (HBNF) (11)] is an 18-kDa secretary protein that is highly conserved among mammalian species. Northern blot analyses have suggested that OSF-1 expression is developmentally regulated, increasing during gestation in many fetal tissues to maximum levels in neonates and then decreasing in most tissues to low or undetectable levels, except in the brain and calvarium, where OSF-1 content remains rather high even in adults (1,5-7,12). OSF-1 is thus believed to play roles in fetal development and in organogenesis and/or maintenance of brain and bone. OSF-1 exhibits approximately 50% sequence identity at the amino acid level with another heparin-binding protein, MK (midkine), a secreted protein rich in cysteine and basic amino acids with a molecular weight of around 13 kDa (13,14). OSF-1 and MK exhibit neurite extension activity (4-7,11,14-16), and both also exhibit weak mitogenic activity toward fibroblasts and PC12 pheochromocytoma cells (3,17). In addition, OSF-1 exhibits mitogenic activity toward capillary endothelial cells (2), and is secreted from breast cancer cells and functions as a tumor growth factor (18,19). OSF-1 may thus possess tumorigenic and mitogenic activities in addition to its neurotrophic activity.

We have reported the genomic structure of the mouse OSF-1 gene, in which the open reading frame (ORF)

[1] All correspondence to M. Sato Molecular Medicine Research Center, The Institute of Medical Sciences, Tokai University, Bohseidai, Isehara, Kanagawa 259-11, Japan. Fax: +81/463-96-2892. E-mail: masasato@is.icc.u-tokai.ac.jp.

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spans four exons and at least two additional 5′-untranslated region (UTR) exons (upstream exon U2 and downstream exon U1) exist (20). Comparison of the murine genomic structures of MK (21) and OSF-1 (20,22) revealed that these genes have the same exon-intron arrangement, similarly- or identically-sized exons and highly homologous sequences in exons. These findings strongly suggest that MK and OSF-1 are derived from a common ancestral gene (15,20,22).

We have isolated two types of OSF-1 cDNAs which differ at the 5′ UTR sequences: one has a sequence for U1 and U2, while the other has a sequence for U1 (20). These two types of OSF-1 mRNAs are therefore thought to be generated by the use of alternative promoters and splicing of the intron (hereafter referred to as intron -1) between U1 and U2. The mouse OSF-1 gene has two promoters which have been tentatively termed promoters I and II (20). Promoter II is in U2, while promoter I is in intron-1, which comprises 520 bp between U2 and U1. In promoter I, there are one CCAAT-box, two TATA-boxes, a sequence similar to the thyroid hormone-responsive element, one cAMP-responsive element and one Sp1-binding site (20). In addition, a sequence similar to the glucocorticoid-responsive element, one CCAAT-box and one TATA-box are found in promoter II (20). However, we were unable to identify the transcription start site in the mouse OSF-1 gene despite extensive study (20), although in humans transcription initiation sites for OSF-1 mRNA have been mapped within promoter I using primer extension or S1 nuclease protection assay (23-25).

We attempted to determine which OSF-1 promoters are used during murine development and in various cell lines. We employed RT-PCR (reverse transcription-polymerase chain reaction) using specific primers corresponding to either promoter I or II, and demonstrated that (i) promoter I-specific OSF-1 mRNA was widely distributed in the adult tissues examined, and (ii) promoter II was active in highly restricted tissues including calvarium (at an early stage of development) and brain, and in a few cell lines among those examined.

MATERIALS AND METHODS

Animals and cell lines. B6C3F1 (a hybrid of C57BL/6N and C3H/HeN) mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) and bred in our laboratory. From these mice at age 2 months, the calvarium, brain, kidney, intestine, heart, spleen, thymus, testis, seminal vesicle, preputial gland, caput epididymis, cauda epididymis, lung, skeletal muscle, bladder, uterus, ovary and liver were obtained and immediately frozen on dry ice and stored at −70°C until use. Calvaria from mice aged 0.5 months were also dissected. From E-19 embryos [the day when a vaginal plug was observed was designated as embryonic day 0 (E-0)], brain, kidney, intestine and liver were dissected. Brains were obtained from fetuses (E-14), young mice (aged 8 days and 1.5 months) and adult mice (aged 4 and 12 months). The cell lines used were ROS17/2 (26), a rat osteosarcoma cell line; UMR106 (27), a rat osteosarcoma cell line; NIH3T3, C_2Cl_2, a mouse myoblast cell line (28); MC3T3-E1, a mouse osteoblast cell line (29); C3H10T1/2, a mouse fibroblast cell line (30); and Ltk^-, a mouse fibroblast cell line lacking thymidine kinase activity (31). These cells were cultivated in conditions as previously described (32) and harvested at subconfluency. In the case of the MC3T3-E1 cell line, cells were harvested at 1, 7 and 12 days after culture. Total RNA was isolated by the method of Chomczynski and Sacchi (33) using ISOGEN reagent (Wako Pure Chemical Industry, Tokyo, Japan).

RT-PCR analysis of specific mRNAs in mouse tissues. cDNA was prepared from total RNA (4 μg) by reverse transcription (RT) with the following buffer and conditions: RT buffer (GIBCO BRL, Grand Island, NY) containing 50 mM Tris-Cl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 20 mM dithiothreitol, 10 U RNasin (Promega, Madison, WI), 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 10 pmol each of reverse primer, KPP-10 and mβA-RV, and 200 U MMLV-reverse transcriptase (GIBCO BRL) in a final volume of 20 μl, with incubation for 1 h at 42°C. The cDNA solution (3 μl) was mixed with 17 μl of PCR buffer [final concentrations in a 20 μl reaction solution: 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 5 pmol each of the forward and reverse primers, 1 U *Taq* DNA polymerase (Takara Shuzo Co. Ltd., Kyoto, Japan), and 0.2 mM each of dATP, dCTP, dGTP, and dTTP], and was then amplified using a Perkin-Elmer Cetus thermal cycler (Model 9600; Norwalk, CT). The PCR conditions with 24 cycles (except for amplification of mouse β-actin mRNA, for which 15 cycles were employed) were denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min. Preliminary studies showed that use of 24 cycles was preferable, since the amplified products were at the linear stage prior to plateau (data not shown). The PCR primer set for detection of mouse OSF-1 mRNA derived from transcription from the promoter II was as follows: forward primer II-S, 5′-GAT CTG TCA CTC CTA GTG ACA-3′, corresponded to the mouse OSF-1 gene sequence (20) from nucleotides −562 to −542 (present at the 3′ portion of promoter I; Fig. 1), while the reverse primer KPP-10, 5′-ACG CAC ACA CTC CAC TGC CAT TCT CCA CAG-3′, was complementary to nucleotides 381-352 (present at the 5′ portion of ORF; Fig. 1) of mouse OSF-1 cDNA (1). The PCR primer set for detection of mouse OSF-1 mRNA derived from transcription of promoter I was as follows: forward primer I-S, 5′-CTC TAT TTC CCT CCC CGG CAG-3′, corresponded to the mouse OSF-1 gene sequence (20) from nucleotides −5 to +16 (present at the 3′ portion of promoter I; Fig. 1), and KPP-10. The PCR primer set (mβA-S and mβA-RV) for detection of mouse β-actin mRNA was used as previously described (34). Each primer set was designed to produce a PCR product that included an intron-exon border, thereby eliminating the possibility that DNA contamination was responsible for the resulting target products present in the tissue cDNAs. The resulting products (10 μl) were subjected to electrophoresis in 2% agarose gels and stained with ethidium bromide (EtBr). After staining with EtBr, gels were blotted onto GeneScreen*Plus*™ (NEN, Boston, MA) nylon filter membranes for Southern blot hybridization analysis, as previously described (35). The whole cDNA (approximately 1.5 kb) for mouse OSF-1 (1) was used for detection of mouse OSF-1 mRNA as a probe.

Sequencing of the PCR-amplified products. PCR products resulting from the amplification of OSF-1 mRNAs in cDNAs prepared from brains of mice aged 0.5 months or from MC3T3-E1 cells cultured for 7 days were sequenced to ensure that the PCR products corresponded to OSF-1. The PCR products were first purified from 2% agarose gels. Purified PCR products were inserted into the pBluescript SK(−) cloning vector (Stratagene, La Jolla, CA). Sequencing of the PCR product insert was carried out on Qiagen-preparations of plasmid DNA using primers corresponding to the T3 and T7 recognition sites of the vector. DNA sequences were determined by an automated DNA sequencer (Model 310, Applied Biosystems, Inc., Alameda, CA) with the ABI Prism™ dye primer (Applied Biosystems, Inc.).

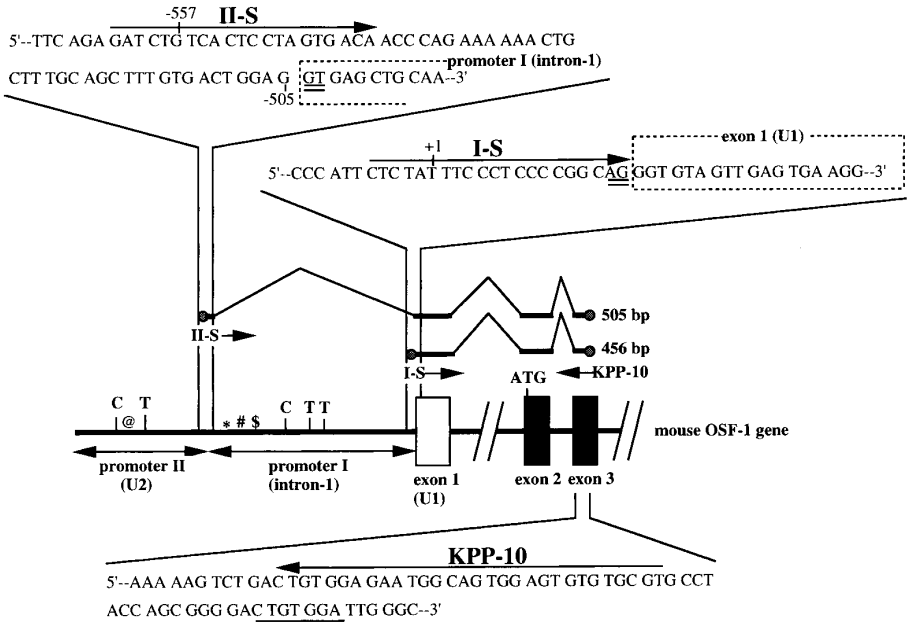

FIG. 1. A diagram showing the 5′ region of OSF-1 gene containing promoter II (U2), promoter I (intron -1), exon 1 (U1), exon 2 (containing ATG) and exon 3. Sense primers I-S and II-S, which recognize the sequences of promoters I and II, respectively, are shown. Reverse primer KPP-10 which recognizes the sequence in exon 3 of OSF-1 gene is also shown. A sequence (5′-CTG TGG A-3′; underlined) probably recognized by KPP-10 primer is present 16 bp downstream of the 3′ end [nucleotide 381; (1)] of the site to which KPP-10 binds theoretically. Closed boxes indicate coding sequences for OSF-1. RT-PCR of OSF-1 mRNA using the I-S/KPP-10 and II-S/KPP-10 primer sets is expected to yield 456 bp and 505 bp products, respectively. The double underlines indicate the consensus sequences for splicing acceptor-donor sites (20). Nucleotide T is designated as +1, the site of which is at the 5′ end of the longest cDNA clone, MC034 (1), derived from transcription from promoter I (20). Motifs of regulatory elements on the promoter regions of mouse OSF-1 gene are identical by symbols as follows; C for CCATT-box, T for TATA-box, @ for a sequence similar to the glucocorticoid-responsive element, * for cAMP-responsive element, # for a sequence similar to the thyroid hormone-responsive element, and $ for Sp1-binding site (20).

RESULTS

Expression of OSF-1 mRNAs in adult calvarium and ovary using I-S/KPP-10 or II-S/KPP-10 primer set. As a preliminary test, we first attempted to amplify OSF-1 mRNAs derived from promoter I or II using RNAs from adult calvarium, a tissue highly enriched with OSF-1 mRNA, and adult ovary, a tissue that does not express OSF-1 mRNA at Northern level (data not shown). As shown in Fig. 2, the calvarium expressed exclusively OSF-1 mRNA from promoter I when a I-S/KPP-10 primer set was used. The size of the amplified product was 456 bp, as expected (EtBr staining in Fig. 2). A similar result was obtained with amplification of adult ovary mRNA, though the amount of RT-PCR product was much lower than for calvarium (EtBr staining in Fig. 2). On the other hand, RT-PCR using the II-S/KPP-10 primer set yielded no significant products from calvarium or ovary mRNAs (EtBr staining in Fig. 2). After staining with EtBr, the gel was blotted onto a nylon filter. When this filter was probed with mouse OSF-1 cDNA, the intensity of a 456 bp band observed on EtBr staining was strongly enhanced (PCR-Southern in Fig. 2). It should be noted that another RT-PCR fragment with 490 bp was observed in the ovary sample after RT-PCR with the I-S/KPP-10 primer set (PCR-Southern in Fig. 2). This 490 bp fragment was found to be generated by addition of a 34 bp short sequence to the 3′ end of the 456 bp fragment, as described below. No hybridizable signals were found when RT-PCR using the II-S/KPP-10 primer set was applied to mRNAs of calvarium and ovary (PCR-Southern in Fig. 2). Based on experiments, a more sensitive method of PCR-Southern analysis was used for further analysis.

Expression of OSF-1 mRNAs in adult organs. When Southern blot analysis of the PCR-amplified products was carried out using the I-S/KPP-10 primer set with adult organs, brain was found to have large amounts of OSF-1 mRNA (Fig. 3, upper column), as expected from results of Northern blot analysis (1). Other organs including intestine, heart, spleen, thymus, kidney, liver, testis, seminal vesicle, preputial gland, cauda epididymis, lung, skeletal muscle and uterus had smaller amounts of OSF-1 mRNA (Fig. 3, upper column). Two kinds of transcripts, an expected transcript with 456 bp and a transcript with 490 bp, were observed in these organs, except in liver and lung, where only the 490 bp mRNA was observed. However, the latter case did not

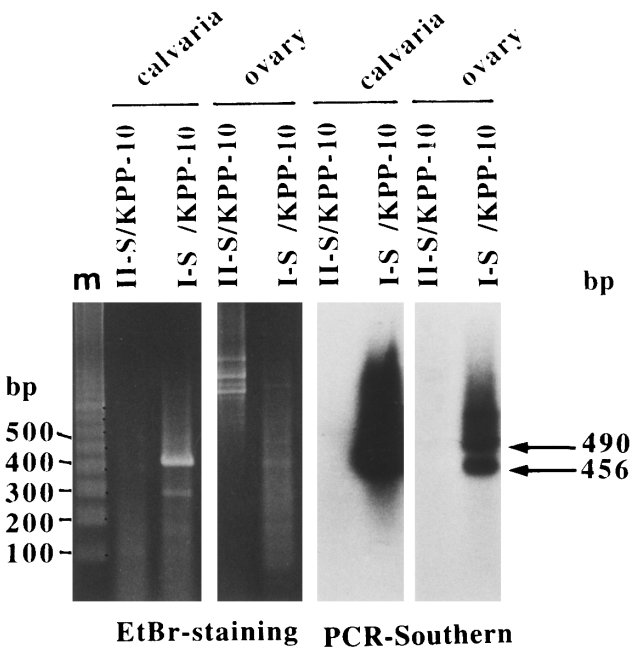

FIG. 2. Analysis of RT-PCR products from the mRNAs of calvarium and ovary of B6C3F1 mice aged 2 months. Total RNA was isolated and reverse-transcribed with KPP-10 and mβA-RV. PCR reaction was performed with a I-S/KPP-10 or II-S/KPP-10 primer set. The products were separated in a 2% agarose gel and stained with EtBr (EtBr-staining). The gel was then blotted onto a filter for Southern blotting (PCR-Southern) using mouse OSF-1 cDNA probe. PCR-Southern analysis was found to be more sensitive than EtBr staining. An expected product with 456 bp was observed in these two tissues when I-S/KPP-10 primers were used. An additional product with 490 bp which was not detected in EtBr-stained gel was also observed after Southern blotting. m, 100-bp ladder markers from Pharmacia.

mean the absence of OSF-1 mRNA expression in liver and lung, since we had observed weak expression of OSF-1 mRNA in those organs using another primer set which could recognize the coding region of OSF-1 cDNA (data not shown). Densitometric scanning of the 456 bp band from brain, intestine and kidney revealed that the amounts of transcript of the 456 bp product in the brain were approximately 74- and 16-fold higher than those in the intestine and kidney, respectively. In contrast to these findings, RT-PCR using the II-S/KPP-10 primer set did not yield a clear hybridizable band in any organ examined except brain, where a very slight signal for an expected product with 505 bp was observed (Fig. 3, middle column). Mouse β-actin mRNA was present in a high degree of abundance in each tissue, as readily observed in EtBr-stained gels, showing that the RNAs examined had been processed in an intact form (Fig.3, lower column). To examine whether the amplified RT-PCR products corresponded to the OSF-1 mRNA, the 456 and 490 bp products obtained from RT-PCR of brain (aged 0.5 months) mRNA or the 505 bp products obtained from RT-PCR of MC3T3-E1 (cultured for 7 days) mRNA were each subcloned into pBluescript SK(−) vector and sequenced. In this fashion, we confirmed that the amplified 456 or 505 bp product accurately reflects mouse OSF-1 mRNA generated from transcription of promoter I or II, respectively (data not shown). However, the amplified 490 bp product possessed an additional 34 bp sequence (5′-GCC TAC CAG CGG GGA CTG TGG AGA ATG GCA GTG G-3′) at the 3′ end of the 456 bp product. This short sequence was thought to be generated by differential binding of KPP-10 reverse primer to the OSF-1 mRNA, since a sequence (5′-CTG TGG A-3′) recognized by KPP-10 primer was present 16 bp downstream of the 3′ end [nucleotide 381; (1)] of the site to which KPP-10 binds theoretically (Fig. 1).

Expression of OSF-1 mRNAs in embryonic organs, aged brains and cell lines. We determined the expression of OSF-1 mRNAs in the organs (including liver, kidney and intestine) of E-19 embryos together with the calvarium at 0.5 months of age, since these embryonic organs are known to exhibit high levels of expression of OSF-1 mRNA in mice (36) and rats (5) by Northern blot analysis, but OSF-1 mRNA expression in these organs is significantly reduced in adult mice except in brain (1). In agreement with these findings, we found increased production of the promoter I-specific transcripts in the E-19 organs, but dramatic reduction in levels of these transcripts at the adult stage (2 months after birth) (Fig. 4A). A similar tendency was observed for calvarium (Fig. 4A), in which the OSF-1 gene is known to be actively transcribed (1). In contrast to these findings, production of the promoter II-specific transcript was quite minimal in these embryonic organs, except in calvarium isolated at 0.5 months of age, in which only slight production was observed (Fig. 4A). These findings suggest that usage of promoter I is predominant even in embryonic mice.

We next examined the synthesis of OSF-1 mRNAs in brains at various stages (E-14, 8 days, 1.5, 4 and 12 months after birth), since OSF-1 mRNA is most abundant in brain among organs examined (1) and is known to function as a neurotrophic factor (4-7,11,15,16). In mouse brain, the promoter I-specific OSF-1 mRNA was highly expressed and appeared to be equally present throughout development, whereas the levels of promoter II-specific OSF-1 mRNA varied depending on the stage, with peaks at 8 days and 12 months of age, although those levels were much lower than that of promoter I-specific OSF-1 mRNA in the 2-month-old brain (Fig. 4A).

ST2, MC3T3-E1, C3H10T1/2 and NIH3T3 cells strongly exhibited the promoter I-specific transcript (Fig. 4B, upper column). These findings agree with the previous finding of expression of OSF-1 mRNA in MC3T3-E1 and NIH3T3 cells in a study using Northern blot hybridization (1). The MC3T3-E1 cells also exhibited the promoter II-specific transcript with maximal

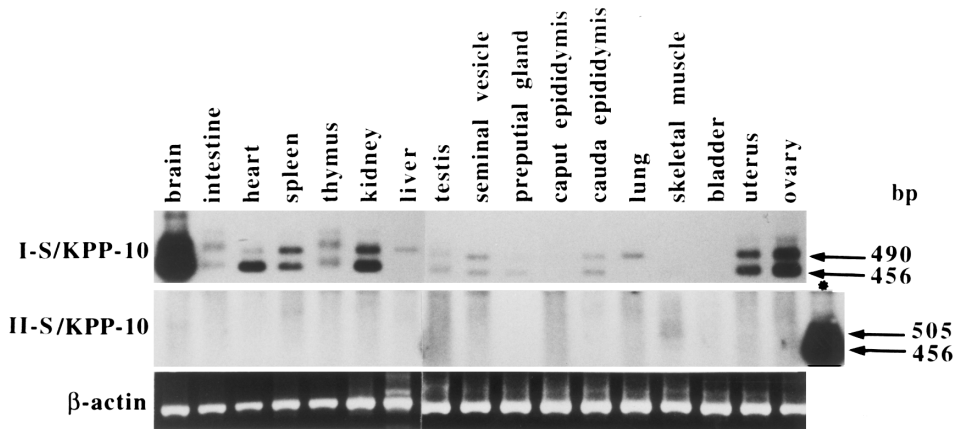

FIG. 3. PCR-Southern analysis of RT-PCR products in the organs of B6C3F1 mice aged 2 months. Total RNA was reverse-transcribed with KPP-10 and mβA-RV. PCR reaction was performed with a I-S/KPP-10, II-S/KPP-10 or mβA-S/mβA-RV primer set and processed as described in **Fig. 2.** An expected product with 456 bp and an additional product with 490 bp were observed in many samples when I-S/KPP-10 primers were used (upper column). RT-PCR using II-S/KPP-10 primer set yielded no clear hybridizable band in any organ examined except brain where a very slight signal with 505 bp was observed (middle column). The asterisk indicates the RT-PCR products from adult brain mRNA obtained using I-S/KPP-10 primer set as a control. Note that the intensity of the RT-PCR product with 505 bp in the adult brain obtained using II-S/KPP-10 primers is much less than that of the RT-PCR product with 456 bp obtained using I-S/KPP-10 primer set (middle column).

levels of this transcript at 7 days after culture (Fig. 4B, middle column). C3H10T1/2 cells also expressed mRNA from transcription of promoter II, but other cells did not (Fig. 4B, middle column).

DISCUSSION

In this study, we used RT-PCR to examine the transcriptional regulatory regions in the 5′-flanking region of the mouse OSF-1 gene, where dual promoters appear to exist. We showed that (i) expression of OSF-1 mRNA is ubiquitous even in the adult stage, though maximal expression occurs in brain and in bone tissues such a calvarium, (ii) OSF-1 mRNAs are generated mainly from transcription at the proximal promoter, i.e., promoter I, and (iii) the usage of the distal promoter, promoter II, is rare throughout murine development, but in some tissues (calvarium at 0.5 months of age, and embryonic and aged brains) and certain cell lines (MC3T3-E1 cells cultured for 7 days and C3H10T1/2) this promoter is functional.

Interestingly, mouse MK is thought to have two promoters, since there are three kinds of MK cDNAs (MK1, MK2 and MK3) (14,37), which share a coding sequence consisting of four exons but have three distinct 5′-UTR sequences corresponding to U1, U2 and U3 of the mouse MK gene (21). The transcriptional initiation sites have been mapped on the proximal promoter region (21) (comprising approximately 180 nucleotides) between U2 and U3 using results of analysis by primer extension assay (37). However, the transcriptional initiation site has not yet been identified in the distal promoter region upstream of the U3 of the MK gene, from which MK3 cDNA is thought to be generated. Given that MK and OSF-1 are structurally highly related proteins, the patterns of usage of the two promoters present in the MK and OSF-1 genes are expected to be similar. In fact, Tomomura et al. (37) reported that MK2 mRNA, a major type of MK mRNA, is generated from the proximal promoter, which appears to correspond to promoter I of the mouse OSF-1 gene. Since all types of MK mRNA are detected without distinction in embryos by *in situ* hybridization (38), it can be inferred that the distal promoter for MK3 mRNA is also active in embryonic mice. The possibility that the distal promoter for MK3 mRNA is functional in mouse embryos may be only different from the case of mouse OSF-1 promoters, since usage of promoter I (and not promoter II) was predominant in the embryonic stage (E-19) of mice (see Fig. 4A).

As noted above, the transcription initiation site for the mouse OSF-1 gene has not yet been determined. On the other hand, in the human OSF-1 gene, transcription initiation sites have been mapped to the region corresponding to intron -1 of the mouse OSF-1 gene, where promoter I exists (23-25). In addition, it was recently demonstrated using RT-PCR that the human OSF-1 gene (h*PTN*) has an additional 5′-exon U2 sequence, in which a promoter corresponding to promoter II of the mouse OSF-1 gene is thought to exist (39). Lai et al. (39) provided evidence that the sequence corresponding to promoter I (intron -1) of the mouse OSF-1 gene is spliced out upon transcription from the U2 region of h*PTN* (39). Thus, the organization of the human OSF-1 gene appears to be quite similar to that of the mouse OSF-1 gene.

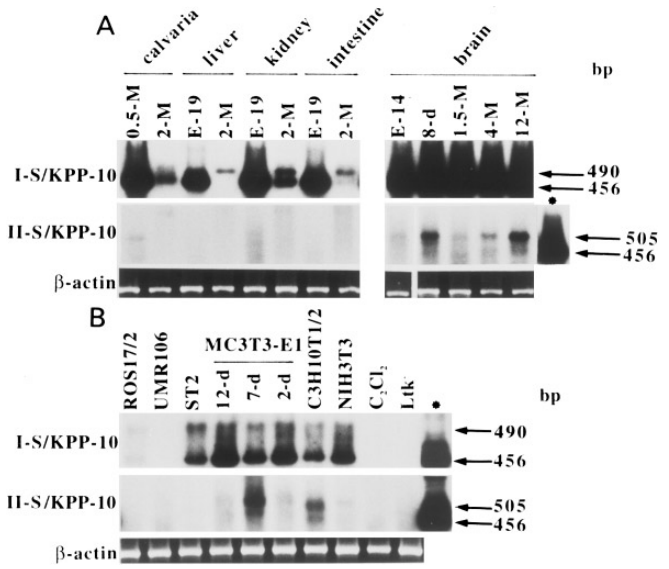

FIG. 4. PCR-Southern analysis of RT-PCR products in the embryonic organs, brains at various stages [E-14, 8-d (8 days after birth), and 1.5-M (months), 4-M and 12-M after birth] and cell lines. Total RNA was reverse-transcribed with KPP-10 and mβA-RV. PCR reaction was performed with a I-S/KPP-10, II-S/KPP-10 or mβA-S/mβA-RV primer set and processed as described in **Fig. 2.** (**A**) RT-PCR products from embryonic tissues (E-19) together with calvaria (at 0.5 months of age) and aged brains of B6C3F1 mice. Note that large amounts of promoter I-specific OSF-1 mRNA are present in each embryonic tissue, but that levels are dramatically reduced in the adult stage (2 months after birth), while the level of promoter II-specific OSF-1 mRNA is extremely low in all stages examined; only the calvaria at 0.5 months of age exhibits slight expression of the promoter II-specific OSF-1 transcript. In the aged brains, promoter I-specific OSF-1 mRNA is highly expressed and appears to be equally present throughout stages, whereas levels of promoter II-specific OSF-1 mRNA varied depending on the stage, with peaks at 8 days and 12 months of age. (**B**) RT-PCR products from cell lines. MC3T3-E1 cells were cultured for 2, 7 and 12 days and then harvested for RT-PCR analysis. Note that MC3T3-E1 cells exhibit maximal production of promoter II-specific transcript after 7 days of culture. Asterisks indicate the RT-PCR products from the adult brain mRNA obtained using I-S/KPP-10 primer set as a control.

We found that usage of promoter II is rare throughout murine development, and that only a few tissues including brain and calvarium (at early but not later stages of development) exhibit relatively high degrees of expression of the promoter II-specific transcript. Interestingly, the osteoblastic cell line MC3T3-E1 when cultured for 7 days exhibited the highest level of expression of the promoter II-specific transcript (see Fig. 4B, middle column). Since MC3T3-E1 cells can differentiate into calcified cells *in vitro* on simple cultivation without cell passage (40), it appears that the MC3T3-E1 cells cultured for 7 days may correspond to the osteoblasts found in calvarium at 0.5 months of age, where promoter II is relatively active (see Fig. 4A).

Genes of various species have been shown to carry dual promoters, selective usage of which results in transcripts that carry different 5′-non-coding sequences but identical protein coding sequences. Examples include *Drosophila Antennapedia* (41,42) and human *lck* (43). The two promoters of *Drosophila Antennapedia* are differentially regulated during embryogenesis (44). For human *lck*, increased usage of the 3′ promoter is observed in malignant cell lines (45). The functional roles of the OSF-1 dual promoters can be studied by examining how these promoters contribute to murine embryogenesis, organogenesis, maintenance of organ function and malignant transformation.

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