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Molecular cloning and functional characterization of the mouse *mafB* gene

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Abstract

The Maf family of the transcription factors plays a pivotal role in controlling development and cellular differentiation. To clarify the molecular mechanisms controlling *mafB* expression, a genomic clone of the mouse *mafB* gene was isolated and analyzed. RNase protection analysis determined the transcription initiation site at 389 bp upstream from the translation initiation site. The 3′ end of the gene is located at 946 bp downstream from the termination codon. The gene lacks intron structure. Sequence analysis showed a TATA-like sequence (5′-GATAAAA-3′) and an inverted CCAAT-box (5′-ATTGG-3′) in the promoter region. Upstream of these sequences, there are several potential regulatory elements, including two GC-boxes (5′-GGGCGG-3′), and a palindromic sequence (5′-GTCAGCTGAC-3′) which contains two Maf recognition elements (MARE, 5′-GCTGAC-3′) and an E-box (5′-CAGCTG-3′).

Transient transfection analysis with the 5′-flanking region of the *mafB* gene demonstrated that these elements are important for *mafB* gene expression. In addition, cotransfection analysis indicated that the MyoD activates the mouse *mafB* promoter and the gene is positively auto-regulated by its own product.

Keywords: Luciferase-assay; *mafB*; MyoD; Oncogene; Transcription factors

1. Introduction

The *v-maf* oncogene was identified from the genome of avian transforming retrovirus (AS42) (Nishizawa et al., 1989; Kawai et al., 1992). *v-maf* encodes a transcription factor containing the typical bZip structure, a motif for protein dimerization and DNA binding, and an acidic transactivation domain (Landschulz et al., 1988; Vinson et al., 1989). So far, a number of cellular *maf* family genes have been identified including *mafB* (Kataoka et al., 1994a), *c-maf* (Nishizawa et al., 1989), *mafK*, *mafG*, *mafF* (Fujiwara et al., 1993; Kataoka et al., 1995), *Nrl* (Swaroop et al., 1992), *L-maf* (Ogino and Yasuda, 1998), and *mafA* (Benkhelifa et al., 1998).

Kataoka et al. (1994b) identified the v-Maf binding DNA sequence, Maf recognition element (MARE, 5′-TGCTGACTCAGCA-3′). The MARE sequence overlaps with the 12-*o*-tetradecanoylphorbol-13-acetate (TPA) responsive element (TRE, 5′-TGACTCA-3′), and cyclic AMP responsive element (CRE, 5′-TGACGTCA-3′). Recently, Matsushima-Hibiya et al. (1998) showed that Maf-1 (a rat homolog of MafB) can firmly bind to the first half sequence of MARE (5′-GCTGAC-3′), and activates its transcriptional activity. v-Maf can form homodimers as well as heterodimers with both Jun and Fos. MafB forms dimers with itself, v-Maf, and Fos, but not with Jun (Kataoka et al., 1994b; Matsushima-Hibiya et al., 1998). These results indicate that the Maf family of transcription factors mediates a wide variety of transcriptional regulations.

A *Kreisler* mutant mice study indicates an important role for *mafB* (*Kreisler* gene, *Kr*) in the segmentation of the hindbrain during early development (Cordes and Barsh, 1994). Manzanares et al. (1997) reported that the product of the *Kr* gene directly activates the expression of *Hoxb-3* gene in rhombomere 5 in the mouse. It was found that MafB interacts with Ets-1, transcription factor containing a helix-turn-helix DNA binding

Abbreviations: bp, base pairs; bZip, basic and leucine zipper; DMEM, Dulbecco's modified minimal essential medium; kb, kilobases; PCR, polymerase chain reaction; RT–PCR, reverse transcription and polymerase chain reaction.

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domain, and inhibits its transactivation activity, thus interrupting erythroid differentiation (Sieweke et al., 1996).

As previously reported, the rat *maf-1* (*mafB*) and *maf-2* (*c-maf*) are expressed in cartilage, lens and spinal cord in a differentiation specific manner (Sakai et al., 1997; Yoshida et al., 1997). These results indicate that the Maf family protein plays an important role in morphogenic processes and cellular differentiation.

For better understanding of the control of *mafB* expression and its involvement in morphogenic processes and cellular differentiation, we have cloned the mouse *mafB* gene, characterized its *cis*-regulatory elements and elucidated the molecular mechanisms for its transcriptional regulation.

2. Materials and methods

2.1. Cloning of the mouse mafB gene and nucleotide sequencing

A genomic mouse DNA library (129SVJ strain), constructed in the lambda FixII phage vector (Stratagene, San Diego, CA), was screened by the rat *maf-1* (rat homolog of *mafB*) cDNA (Sakai et al., 1997). A positive clone was isolated and subcloned to a plasmid vector according to the standard protocol (Sambrook et al., 1989). The nucleotide sequences of the cloned fragments were determined using an ABI Prism dye terminator cycle sequencing kit and ABI 373S DNA sequencer (Applied Biosystems, CA).

2.2. RNase protection analysis

Total cellular RNA was prepared from the cartilage cells, induced by subcutaneous implantation of bone morphogenic protein (BMP) with fibrous glass membrane (Sakai et al., 1997). An RNA extraction kit, ISOGEN (Nippon Gene, Toyama, Japan), was used according to the manufacturer's recommendations. RNase protection analysis was performed as described (Sakai et al., 1997). To construct the template plasmid for synthesis of the riboprobe, a DNA fragment containing the transcription initiation site (−91 to +134) was subcloned into pBluescript II (Stratagene, San Diego, CA). This DNA fragment was prepared by PCR using forward primer (5′-ATGGATCCAGCCATTGGCCAACA-3′) and reverse primer (5′-TTACTAGTAGGGTAGAGAAGCCGGCGA-3′).

An antisense riboprobe was prepared from linearized template with T7 RNA polymerase and [α-^{32}P]UTP.

2.3. Mapping of the 3′ end

To identify the 3′ end of the *mafB* mRNA, 3′-RACE (rapid amplification of cDNA ends) was performed using a RACE kit (Boehringer Mannheim, Germany). The cDNA was synthesized using the total RNA from BMP induced cartilage cells with oligo(dT)-anchor primer (5′-GACCACGCGTATCGATGTCGAC$(T)_{16}$-A/C/G-3′). The cDNA was amplified by PCR with sense primer (5′-CCTGCATGCTGGACATGTATG-3′, 649 nucleotides down stream from the termination codon) and oligo(dT)-anchor primer. The PCR products were amplified again (nested PCR) with internal sense primer (5′-CCATCTTGAGAAGGTAGCAGC-3′, 849 nucleotides from TGA) and oligo(dT)-anchor primer. The products from the second PCR were subcloned into pGEM T-Easy vector (Promega, Madison WI) and sequenced.

2.4. Construction of plasmids

To construct the luciferase reporter gene (−6500/Luc), a 6.9 kb fragment spanning −6.5 kb to +364 relative to the transcription initiation site of the gene, was inserted into a promoter-less luciferase vector, pGVB2 (Nippon Gene, Toyama, Japan). A series of 5′ deletions fused to the luciferase gene was constructed from −6500/Luc using appropriate restriction enzymes or PCR.

To create a MafB expression plasmid (pAct/MafB), the genomic DNA fragment containing the MafB coding region (Fig. 2, −59 to +2016 bp), was inserted into the expression vector (pAct2) driven by the human β-actin gene promoter and enhancer (Gunning et al., 1987).

For the construction of MyoD expression plasmid, MyoD cDNA was produced by RT–PCR with forward primer (5′-ACGGATCCACCATGGAGCTACTATCGCCGC-3′) and reverse primer (5′-GGAAGCTTCAGAGCACCTGGTAAATCG-3′) using rat muscle total RNA as a template. This cDNA was inserted into the pAct2 expression vector (pAct/MyoD).

2.5. Cell culture, transfection and reporter assays

The human hepatoma cell line, HuH-7, was grown in a chemically defined medium ISE-RPMI (Nakabayashi et al., 1982) supplemented with 1% fetal bovine serum (FBS). 24 h before transfection, the cells were plated at a density of 1×10^6 cells/plate in DMEM (Nissui, Tokyo) containing 10% FBS. A total of 5 μg DNA including 1 μg reporter plasmid, 0.5 μg of β-galactosidase expression plasmid (pSV-β-gal, Promega, Madison, WI) as an internal control, with or without 1 μg of expression plasmid of effector gene, and pUC18 DNA was co-transfected into HuH-7 cells. Transient transfection experiments were performed by

the calcium phosphate co-precipitation method according to Chen and Okayama (1987). After 45 h incubation, cells were harvested and extracted in 100 μl of lysis buffer (Nippon Gene, Toyama, Japan) by freezing and thawing. The cell extracts were assayed for luciferase activity using Luciferase Assay System (Nippon Gene, Toyama, Japan) and for β-galactosidase activity (Sambrook et al., 1989). Luciferase activity was standardized using β-galactosidase activity, and all experiments were repeated at least twice.

3. Results and discussion

3.1. Cloning and sequence analysis of the mafB gene

Approximately 1×10^6 recombinants of the mouse genomic library were screened and one positive clone, containing an insert of about 16 kb, was obtained (λ-*mafB*). Fig. 1 shows a restriction enzyme map of the clone. The nucleotide sequence of mouse *mafB* gene including the 5′- and 3′-flanking regions were analyzed (Fig. 2). Comparison of the nucleotide sequences of mouse *mafB* gene with that of the cDNA reported by Cordes and Barsh (1994) showed no difference between corresponding regions.

To identify the transcription initiation site of the *mafB* gene, RNase protection analysis was performed using the antisense riboprobe as described in Materials and methods. As shown in Fig. 3A, an RNase protected band about 130 nucleotide long was detected. This site corresponds to this sequence (5′-CACAGCT-3′) which is located 390 bp upstream from the translation initiation site (Fig. 2). Almost all eukaryotic mRNA start with adenosine, and there is a weak consensus in the surrounding region of the transcription initiation site. Since the second 'A' of this sequence (5′-CACAGCT-3′) almost matches the consensus sequence (Bucher, 1990, 5′-C-A-(G/T/C)-(T/C/A)-(C/T)-(T/G/C)-(T/C) for −1 to +6 bp), it was tentatively decided that the second 'A' of that sequence is the transcription initiation site (Fig. 2).

The 3′ end of *mafB* mRNA was analyzed using 3′-RACE as described in Materials and methods (Fig. 3B). The mouse *mafB* mRNA is terminated 945 nucleotides downstream from the termination codon. The polyA-signal (AATAAAA) is located at 22 bp upstream from the 3′ end of the gene.

These results, together with sequence data, indicate that the mouse *mafB* mRNA might be 2303 nucleotides long without a polyA tail, and the gene contains no intervening sequence (intron). Kataoka et al. (1994a) have reported that the chicken *mafB* gene is an intronless gene, and we found that the rat and mouse *c-maf* genes are also intron-less (Sakai et al., unpublished).

Database analysis of the 5′ upstream region to the transcription initiation site failed to identify the conventional TATA-box. However, a TATA-like sequence (5′-GATAAA-3′) was found to be located at nucleotide −31 bp referring to the transcription initiation site. Recent reports have shown that the GATAAA element is located within 20–30 bp upstream from the transcription initiation site of several genes, including the minimal promoter (MinL) of the BHLF1 gene derived from Epstein–Barr virus, the human factor VIII gene and the human alpha 6 integrin gene (Lieberman and Berk, 1991; Figueiredo and Brownlee, 1995; Lin et al., 1997).

An inverted CCAAT-box (or Y box) is located at 48 bp upstream from the transcription initiation site. In addition, there are several potential regulatory elements,

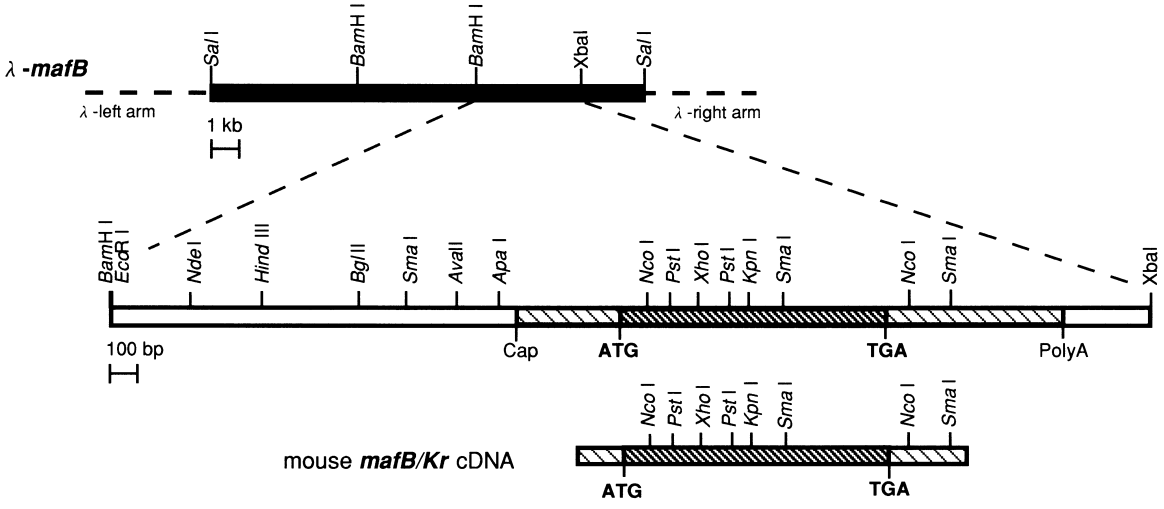

Fig. 1. Restriction endonuclease maps of the mouse *mafB* gene and *mafB*/*Kr* cDNA. Filled boxes denote the insert of the clone. Shadowed area indicates exon with coding region (dark shadow). The mouse *mafB*/*kr* cDNA (Cordes and Barsh, 1994) is also indicated (lowest bar).

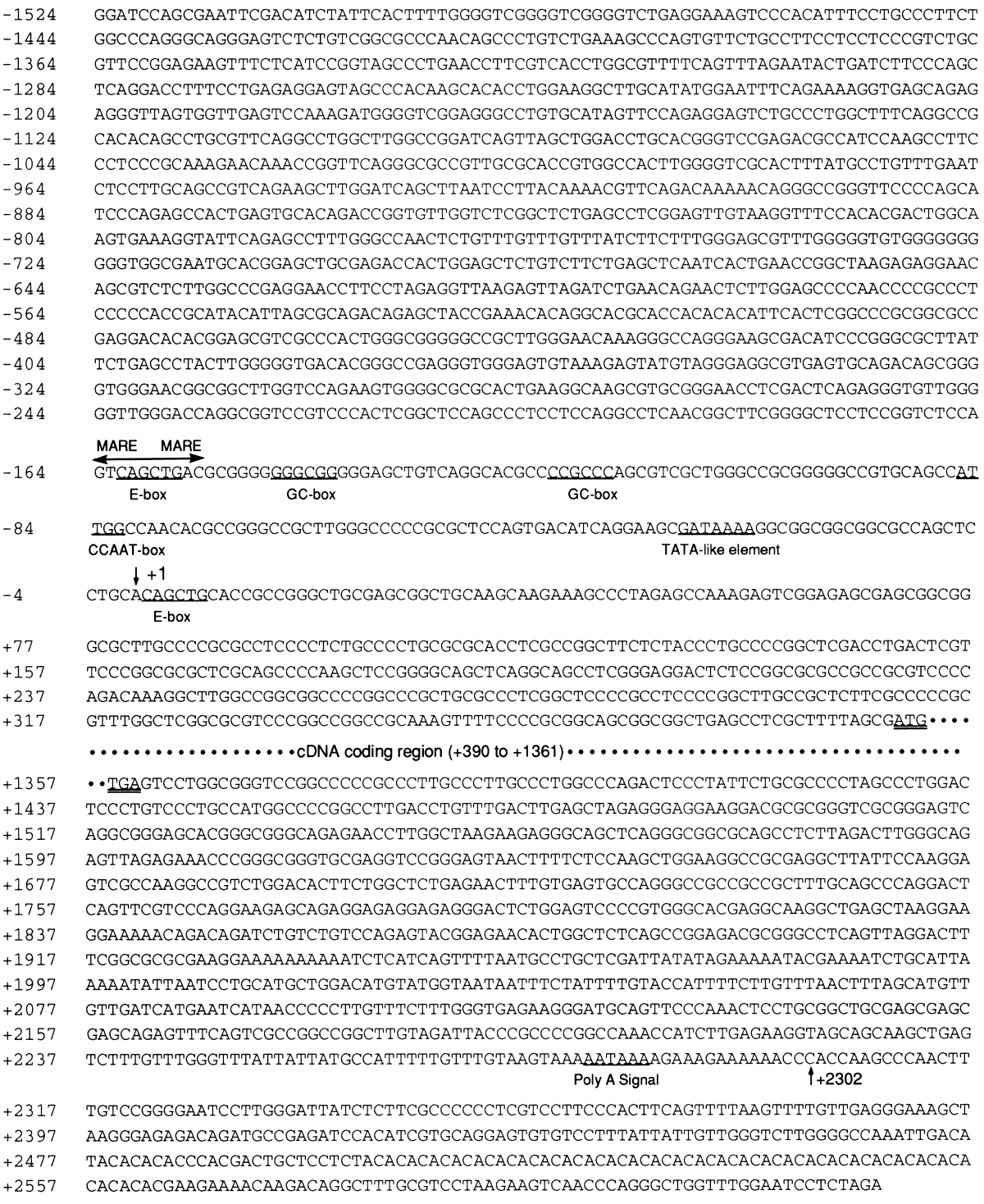

Fig. 2. The nucleotide sequence of the mouse *mafB* gene. The adenosine at the transcription start site is designated +1. The vertical arrows indicate the transcription initiation and the polyA-attached sites. The putative potential binding sites for transcription factors are underlined. Translation initiation codon ATG, termination codon TGA and polyadenylation signal are double underlined. The mouse *mafB* cDNA coding sequence was omitted and marked by dotted line. The nucleotide sequences have been deposited in the GenBank/EMBL/DDBJ databank with accession No. AF180338.

including two GC-boxes (5′-GGGCGG-3′) at −123 and −148 bp, a palindromic sequence (5′-GTCAG-CTGAC-3′) at −164 bp, containing two MARE (Maf recognition element, 5′-GCTGAC-3′) (Matsushima-Hibiya et al., 1998), and an E-box (5′-CAGCTG-3′) and another E-Box at +2 bp. The presence of multiple

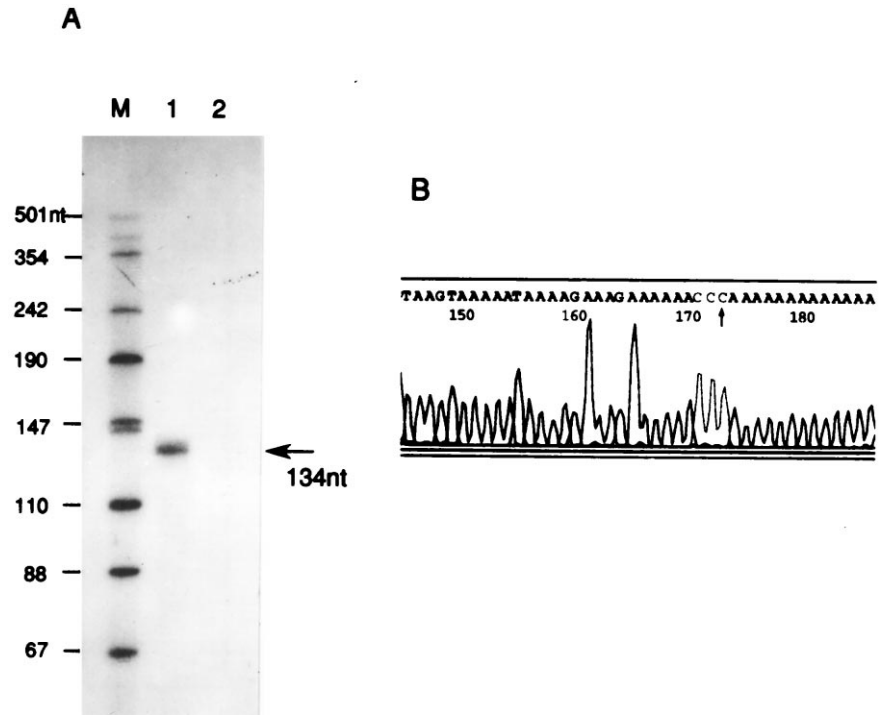

Fig. 3. Determination of 5′ and 3′ ends of the mouse *mafB* mRNA. (A) 30 μg of total RNA from cartilage cell induced by BMP (lane 1) or yeast RNA (lane 2) was hybridized with anti-sense RNA probe (5×10^5 cpm) at 55°C overnight. The hybrids were digested by RNase A (10 μg/ml) at 30°C for 1 h. pUC18 DNA, digested by *Hpa*II and terminally labeled, was used as size marker (lane M). (B) The nucleotide sequence data of 3′-RACE analysis of the mouse *mafB* mRNA. An arrow indicates polyA-attached site.

transcription factor binding sites near the transcription initiation site strongly suggests that this region contains the functional *mafB* gene promoter.

3.2. Functional characterization of the mafB promoter

To determine the transcriptional activity of the *mafB* gene promoter, various lengths of the 5′-flanking sequence were fused to the luciferase reporter gene and introduced into HuH-7 cells by transient transfection method. HuH-7 is a well-differentiated hepatocellular carcinoma cell line which expresses significant amounts of *mafB* mRNA, similar to the mouse and rat liver tissues (Sakai et al., 1997). The results of transfection analyses are shown in Fig. 4. The −6500/Luc has a strong promoter activity, directing 190-fold higher luciferase activity than that of negative control plasmid, pGVB2. The progressive deletion of the distal 5′-flanking region from −6.5 kb to −601 bp resulted in a significant increase (5-fold) in the promoter activity, and led to the assumption that some negative regulatory elements may lie within this region. Further deletion studies from −601 to −229 bp resulted in a significant decrease in the promoter activity. However, a drastic decrease in luciferase activity was observed when the region from −229 to −61 bp was deleted. The activity of promoter function reduced about 95%. These results showed that the region between −229 and −61 bp of the *mafB*gene contains elements essential for directing the transcription activity of the reporter gene in HuH-7 cells and is required for the *mafB* gene basal transcription activity.

As shown in Fig. 2, an inverted CCAAT box (5′-CCATTGGCC-3′) is located at −88 bp upstream from the transcription initiation site. The CCAAT consensus sequence (5′-RRCCAAT(C/G)(A/G)-3′) proposed by Bucher (1990) is well conserved with the CCAAT-box of the *mafB* gene. This suggests that the CCAAT box binding proteins such as C/EBP, CTF/NFI or NF-Y may bind to the CCAAT-box of the *mafB* gene. To examine whether the CCAAT-box could function in the promoter of the *mafB* gene, we compared the −91/Luc, which contains a CCAAT-box and −61/Luc constructs. A drastic decrease in luciferase activity was observed with −61/Luc construct in HuH-7 cells (Fig. 4B). This strongly suggests that CCAAT-box consensus is required for the basal promoter activity of *mafB* gene. This low luciferase activity in the −61/Luc may be due to the relatively poor TATA element activity. In addition to TATA element, other promoter regulatory elements such as CCAAT-box, GC-boxes may play an important role in enhancing the basal promoter activity of this gene. This prediction agrees with the suggestion of Lieberman and Berk, 1991. They stated that the minimal promoter (MinL) of BHLF1 gene (Epstein–

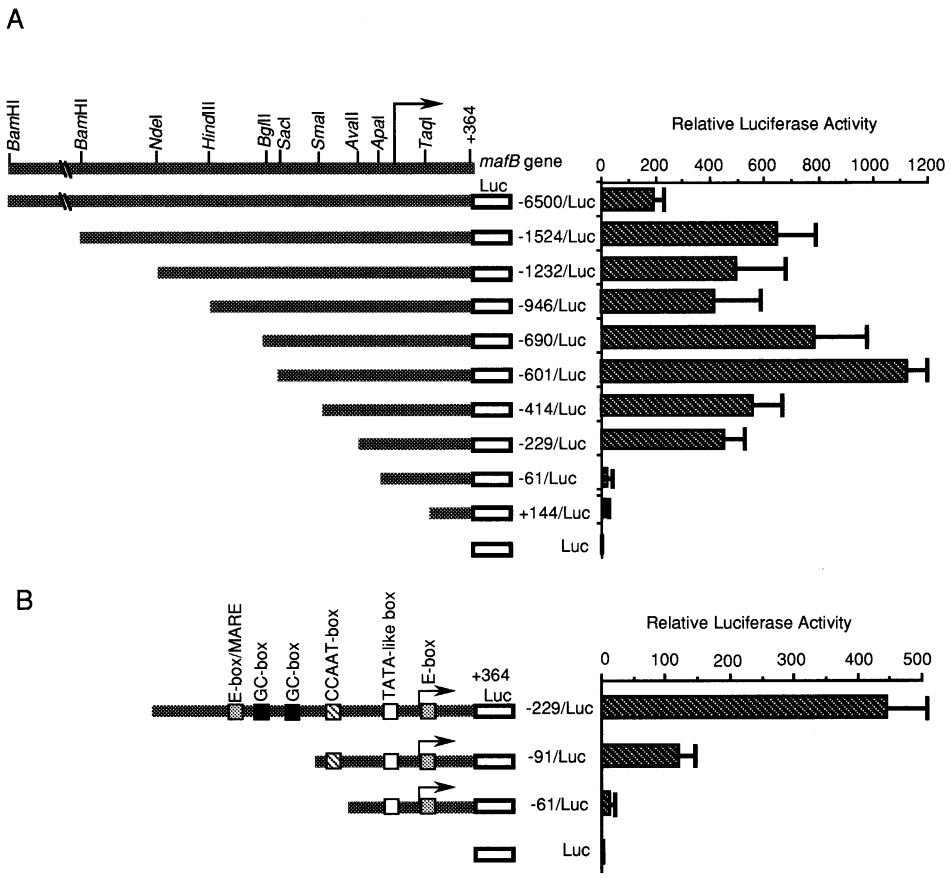

Fig. 4. Promoter activity of deletion constructs. A series of deletion constructs fused to the luciferase gene were transfected into HuH-7 cells. The relative luciferase activities of reporter constructs are shown on the right and are presented as a relative value (-fold) to that of pGVB2 vector (Luc). Bars indicate standard error of the mean. (A) A restriction map representing the *mafB* genomic DNA insert is shown at the top. The numbers of each construct indicate the 5′ end of the promoter fragment relative to the transcription initiation site. (B) The deletion constructs of proximal region. The putative regulatory DNA elements are indicated schematically by boxes.

Barr virus), which contains the 5′-GATAAAA-3′ element, requires upstream factors to stabilize the interaction of TFIID with the promoter.

In the region between −229 and −91 bp of the *mafB* gene promoter, there are two putative GC boxes (5′-GGGCGG-3′), located at nucleotides −148 and −123 bp. In addition, a palindromic sequence containing one putative E-box (5′-CAGCTG-3′) and two MARE (5′-GCTGAC-3′) with opposite orientation are located at nucleotide −164 to −155 bp (Fig. 2). Deletion of sequence from −229 to −91 bp resulted in about 3-fold decrease in the promoter activity (Fig. 4B).

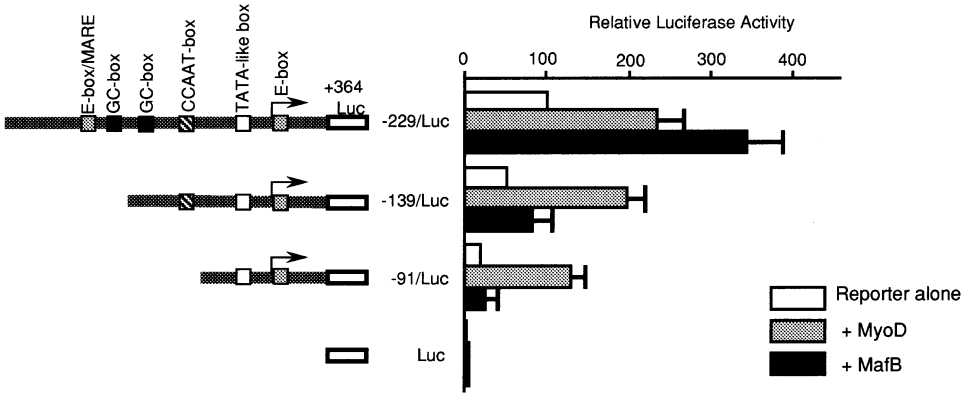

Fig. 5. Effects of MyoD and MafB on the *mafB* gene expression in HuH-7 cells. The luciferase reporter constructs were cotransfected with expression constructs for MyoD or MafB. Luciferase activity is presented as in Fig. 4.

This suggests that the two GC-boxes, E-box and/or MARE function as enhancer elements.

3.3. MyoD and MafB transactivate the mafB gene

The results of the reporter transfection analysis demonstrated that the region between −229 and −61 bp of the *mafB* promoter contains positive regulatory elements essential for directing the transcription of the reporter gene in HuH-7 cells. As described above, potential regulatory elements, MARE and E-box, are located within this region. We also found another E-box element (+2 to +7 bp) which is located near the transcription initiation site of the gene. MyoD was demonstrated to bind the E-box element of the genes involved in myogenesis (Lassar et al., 1989). To demonstrate whether the MyoD could activates the *mafB* promoter, cotransfection experiments were performed in HuH-7 cells. The effector plasmid pAct/MyoD was cotransfected with various reporter constructs. The activities of the −229/Luc, −139/Luc, −91/Luc were stimulated by pAct/MyoD at 2.2, 3.3 and 5.5-fold, respectively (Fig. 5). This result suggests that expression of MyoD in HuH-7 cells can enhance the *mafB* promoter activity through binding to the two E-boxes present in the *maf*B gene promoter. We reported previously that the rat *mafB*/*maf-1* mRNA is high in muscle among the tissues examined (Sakai et al., 1997). However, the expression of chicken *mafB* mRNA is lower in muscle. This is presumably because of the lack of E-box in the chicken *mafB* promoter (Kataoka et al., 1994a). Ou et al. (1994) demonstrated that the E-box might play a role in stabilizing the binding of TFIID. This study suggests that MyoD may be involved in the regulation of the function of mouse *mafB* TATA-like element and thereby promotes the assembly of preinitiation transcriptional complexes.

The palindromic sequence (−164 to −155) contains the two halves of MARE in opposite orientation. Matsushima-Hibiya et al. (1998) showed that MafB could strongly bind to the first half of MARE (5′-GCTGAC-3′) and stimulate its transcriptional activity. To show whether MafB can activate the *mafB* promoter, cotransfection experiments were performed. The effector plasmid pAct/MafB was cotransfected with the reporter constructs. This experiment resulted in 3.3-fold higher luciferase activity than that of −229/Luc without MafB (Fig. 5). The −139/Luc and −91/Luc, in which the MARE sequence was deleted, resulted in only a slight increase of the promoter activity by cotransfection. These findings indicate that the expression of the mouse *mafB* gene is positively autoregulated by its product, MafB, through the MARE sequence. This suggests that by feeding back on its own promoter and stimulating its own transcription, MafB could be responsible for prolonging the transient signals induced by extracellular stimulation.

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