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Genomic structure and analysis of transcriptional regulation of the mouse zinc-fingers and homeoboxes 1 (*ZHX1*) gene

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Abstract

The mouse *zinc-fingers and homeoboxes 1* (*ZHX1*) gene was cloned and its transcriptional regulatory mechanism analysed. The mouse *ZHX1* gene spans approximately 29 kb and consists of five exons. Exons 1–3 contain the nucleotide sequence of the 5′-noncoding region of mouse ZHX1 cDNA, exon 4 contains a part of the 5′-noncoding region, an entire coding sequence, and a part of the 3′-noncoding sequence, and exon 5 contains the resulting 3′-noncoding sequence. The *ZHX1* gene exists as one copy in the haploid mouse genome. Two species of ZHX1 mRNA with or without the nucleotide sequence of the third exon are produced by an alternative splicing. To investigate the regulatory elements involved in the transcription of the *ZHX1* gene, transient DNA transfection experiments with *ZHX1*/firefly luciferase reporter genes were performed using a lipofection method. Functional analyses of a series of 5′- and 3′-deletion constructs of the reporter genes revealed that the nucleotide sequence between −59 and +50 is required for full promoter activity in mouse embryonal carcinoma F9 cells. Two positive regulatory *cis*-acting elements in the region were identified. These elements, designated as Box A and Box B, are located between nucleotides −47 and −42 and +22 and +27, respectively, and synergistically stimulate transcription of the mouse *ZHX1* gene. Electrophoretic mobility shift assays with specific competitors and antibodies show that PEA3 and Yin and Yang 1 (YY1) bind to Box A and Box B, respectively. Thus, we conclude that PEA3 and YY1 synergistically stimulate the transcription of the *ZHX1* gene.

Keywords: Genomic cloning; Nucleotide sequence; Transcription factor; YY1; PEA3

1. Introduction

Gene expression is regulated at several different stages. In most genes, regulation mainly occurs at the transcription level (Fry and Farnham, 1999). Generally, gene transcription is accomplished via interactions between *cis*-acting elements and transcription factors. Transcription factors primarily function by binding to the cognate DNA sequence in the promoter region of the gene. These DNA-binding proteins not only interact with one another, but also interact with DNA-nonbinding proteins to regulate the transcription of the genes. The latter, including co-activators and co-repressors, can function by bridging or interfering with interactions between DNA-binding proteins and the basic transcription machinery (Hu and Lazar, 2000; Vo and Goodman, 2001).

Abbreviations: NF-Y, nuclear factor-Y; ZHX1, zinc-fingers and homeoboxes 1; AD, activation domain; ZF, zinc-finger; HD, homeodomain; IL-2, interleukin-2; RT, reverse transcription; PCR, polymerase chain reaction; EMSAs, electrophoretic mobility shift assays; PAGE, polyacrylamide gel electrophoresis; YY1, Yin and Yang 1; NF-E2, nuclear factor-E2; Stat, signal transducer and activator of transcription; NF-1, nuclear factor-1; AP-1, activator protein-1; AP-2, activator protein-2; C/EBP, CCAAT/Enhancer-binding protein; Oct-1, octamer-binding protein-1; GATA, GATA-binding protein; USF, upstream stimulatory factor; Sp1, specificity protein 1; Egr-1, early growth response gene-1; CBP, cyclic AMP response element-binding protein-binding protein; JAK, Janus kinase.

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Nuclear factor-Y (NF-Y), a ubiquitous transcription factor, recognizes and binds to an inverted CCAAT box (also referred to as the Y box) (Mantovani, 1999). It is composed of three subunits, NF-YA, NF-YB, and NF-YC, all of which are necessary for DNA binding. NF-Y plays an important role in tissue-specific gene expression as well as ubiquitous gene expression (Mantovani, 1999). We previously reported on the cloning of human zinc-fingers and homeoboxes 1 (ZHX1) as a protein that interacts with the activation domain (AD) of the NF-YA (Yamada et al., 1999b). We also cloned rat ZHX1 as one of the ZHX1-interacting proteins using a yeast two-hybrid system, indicating that ZHX1 is able to form at least a homodimer (Hirano et al., 2002). Mouse ZHX1 has independently been reported as a nuclear antigen recognized by the B92 monoclonal antibody that is produced by immunization with a cell lysate of mouse 14F1.1 endothelial-adipose stromal cells (Barthelemy et al., 1996). The human, rat, and mouse ZHX1 all consist of 873 amino acid residues and contain two Cys_2-His_2-type zinc-finger (ZF) motifs and five homeodomains (HDs) (Barthelemy et al., 1996; Yamada et al., 1999b; Hirano et al., 2002). The amino acid sequence of the human ZHX1 showed a 93 and 91% similarity with that of the rat and mouse forms, respectively. Two ZF motifs and five HDs are highly conserved (Barthelemy et al., 1996; Yamada et al., 1999b; Hirano et al., 2002). They belong to the ZF class of the homeobox protein superfamily (Gehring et al., 1994). The amino acid sequence between 272 and 564 which contains HD1 through HD2 region of human ZHX1 is required for interaction with a glutamine-rich AD of the NF-YA (Yamada et al., 1999a). The human *ZHX1* gene is located on chromosome 8q, between markers CHLC.GATA50B06 and CHLC.GATA7G07 (Yamada et al., 1999b). ZHX1 mRNA is expressed ubiquitously (Barthelemy et al., 1996; Yamada et al., 1999b; Hirano et al., 2002). Recently, we reported that ZHX1 functions as a transcriptional repressor localized in the nuclei (Yamada et al., 2002). It has been reported that interleukin-2 (IL-2) induces the expression of ZHX1 mRNA in mouse T cells (Herblot et al., 1999). However, the transcriptional regulatory mechanism of the *ZHX1* gene has not yet been elucidated.

In the present study, as the first step in evaluating the physiological roles of ZHX1, we report on the isolation of the mouse *ZHX1* gene from a 129 svj mouse male spleen genomic library, its entire genomic structure, and an analysis of the transcriptional mechanism of the gene.

2. Materials and methods

2.1. Materials

The TRIZOL reagent, RNase inhibitor, Superscript II, and Lipofectamine PLUS were purchased from Invitrogen (Groningen, the Netherlands). The ExTaq DNA polymerase and BcaBest DNA labeling kit were obtained from Takara Biomedicals (Kyoto, Japan). The Colony/Plaque Screen filter, [α-^{32}P]dCTP (111 TBq/mmol), and [γ-^{32}P]ATP (111 TBq/mmol) were purchased from NEN Life Science Products (Wilmington, DE). Hybond N and Hybond N + were purchased from Amersham Pharmacia Biotech (Cleveland, OH). The pBluescriptII SK + vector and Quikchange site-directed mutagenesis kit were purchased from Stratagene (La Jolla, CA). The BigDye terminator cycle sequencing kit FS was purchased from Applied Biosystems Japan (Tokyo, Japan). The pGEM-T Easy, pGL3-Basic, pGEM-3Zf(+), and dual luciferase reporter assay system were obtained from Promega (Madison, WI). The QIAEXII gel extraction kit, QIAGEN lambda and plasmid kits were purchased from Qiagen (Hilden, Germany). The Bio-Rad protein assay kit was obtained from Bio-Rad (Hercules, CA). Anti -PEA3 (SC-113X), anti-Ets1/Ets2 (SC-112X), anti-Ets2 (SC-351X), and anti-YY1 (SC-1703X) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

2.2. Isolation of total RNA, reverse transcription (RT) and polymerase chain reaction (PCR)

Total RNA was isolated from various tissues or cells using the TRIZOL reagent. RT was performed at 37 °C for 1 h in a 50-μl reaction mixture that contained 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.2 mM dNTPs, 2 mM DTT, 20 units of RNase inhibitor, 200 units of Superscript II, 1 μg of total RNA and 0.1 μg of random hexamer. PCR was then performed using a 50-μl reaction mixture that contained 10 mM Tris–HCl (pH 8.3), 100 mM KCl, 3 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of PCR primers, 2.5 μl of the reverse transcription reaction as a template, and 5 units of ExTaq DNA polymerase. The PCR was conducted at 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min.

2.3. Probe DNAs

Three probes were prepared using the RT–PCR or PCR. For RT–PCR, 1 μg of mouse brain total RNA was used as a starting material. For probe 1, oligonucleotides, TSZHX1S 3, 5′-TCTGAAATGCAAGATGGCGGC-3′ and Cas, 5′-CTGCCATAGCTTCTTCAGCAT-3′ were used for RT–PCR primers. The product was subcloned into the pGEM-T Easy vector. For probe 2, oligonucleotides, mZHX1s, 5′-CG CGGGATCCACAATCTTTGAACAGACAATAAATG-3′ and mZHX1as, 5′-CCGGGAATTCGAGTTCTGTTGAG-CAGACACC-3′ were used for RT–PCR. Amplified DNA was digested with *Eco* RI and *Bam* HI and subcloned into the *Eco* RI/*Bam* HI sites of the pBluescriptII SK + . For probe 3, PCR was carried out with a combination of primers, 1st-down, 5′-GGGAAAGGGCAGTAGAATC-3′ and 1st-up, 5′-GGAGCAGTCAGTGCTCTTA-3′ and pBSII-BX42 (see below) as a template. The product was also subcloned into the pGEM-T Easy vector. After confirming the

nucleotide sequence, the respective inserts were used for probes. These probe DNAs were labeled with [α-^{32}P]dCTP using the BcaBest DNA labeling kit.

2.4. Southern blot analysis

The 129 mouse liver genomic DNA was a gift from Dr. Tamio Noguchi (Nagoya University, Japan). The genomic DNA (10 μg) was digested with *Eco* RI, *Bam* HI, *Pst* I, or *Hin*dIII, subjected to a 0.7% agarose gel electrophoresis, and blotted to a Hybond-N membrane filter. The DNA was fixed on the membrane by UV irradiation. Prehybridization and hybridization were carried out at 65 °C in 5× Denhardt's, 5× SSC, 0.5% SDS, and 200 μg/ml heated salmon sperm DNA without or with ^{32}P-labeled probe 2, respectively. Washing was performed twice at 65 °C for 30 min in 0.1× SSC, 0.1% SDS. After the filter was dried, it was exposed to a Kodak X-AR film at −80 °C with an intensifying screen.

2.5. Genomic library screening

A 129 svj mouse male spleen genomic library was a generous gift from Dr. Makoto Satoh (Fukui Medical University, Japan). Independent clones (5×10^5) were screened by the plaque hybridization method using ^{32}P-labeled ZHX1 probe 1, 2, or 3 (Sambrook and Russell, 2001). Prehybridization, hybridization, and washing conditions were same as those used for the Southern blot analysis. Seven independent clones, #1, #2, #3, #4, #31, #42, and #61, were isolated. Lambda DNA was prepared from these positive clones using the QIAGEN lambda kit. Some clones were digested with various restriction endonucleases and the inserts were subcloned into the pBluescriptII SK + vector. Their nucleotide sequences from the lambda DNAs from phage clones or subcloned plasmids were determined with a DNA sequencer (Applied Biosystems 3100). A 2.5 kb *Xba* I/*Bam* HI fragment from the lambda phage clone #42 was subcloned into the *Xba* I/*Bam* HI sites of the pBluescriptII SK + to produce the pBSII-BX42.

2.6. Nuclease S1 analysis

A 2.9 kb *Eco* RI/*Bam* HI fragment of the lambda phage clone #2 was subcloned into the *Eco* RI/*Bam* HI sites of the pBluescriptII SK + to produce pBSII-EB2. PCR was carried out with a combination of P1 primer, 5′-GCTGGAGCTCCGTGGGGGGCC-3′ and P2 primer, 5′-CCGGAAGCTTGCCTCGCCTCAGCGCCTC-3′ using the pBSII-EB2 as a template. After the product had been digested with *Sac* I/*Hin*dIII, the 138 bp fragment was subcloned into the *Sac* I/*Hin*dIII of the pBluescriptII SK + vector to obtain pBSII/mZHX1SH. Single-strand DNA was prepared from *Escherichia coli* which harbors the pBSII/mZHX1SH plasmid after infection with M13KO4 helper phage. The T7 primer was annealed with the single-strand DNA and ^{32}P-labeled DNA was synthesized in the presence of [α-^{32}P]dCTP by means of the Klenow reaction (Sambrook and Russell, 2001). After the DNA had been digested with *Sac* I and subjected to a 2% agarose gel electrophoresis, a uniformly labeled probe was isolated using the QIAEXII gel extraction kit. The probe was hybridized with total RNA from mouse liver and lung or yeast tRNA, and then digested with nuclease S1 (Sambrook and Russell, 2001). After purification, the products were subjected to a 7% polyacrylamide/7 M urea sequencing gel electrophoresis. The gel was dried, and then exposed to a Fuji X-ray film at −80 °C with an intensifying screen.

2.7. Construction of luciferase plasmids

A *Sac* I/*Hin*dIII fragment of the pBSII/mZHX1SH was subcloned into the *Sac* I/*Hin*dIII sites of the pGL3-Basic and pGEM-3Zf(+) to produce pmZHX1/luc88 and pGEM3/88, respectively. The pBSII-EB2 plasmid was digested with *Sac* I, a 0.7 kb *Sac* I fragment was then subcloned into the *Sac* I site of the pmZHX1/luc88 to produce pmZHX1/luc803. Oligonucleotides mcs1, 5′-AATTCGGATCCCCCGGGCTGCAGAGCT-3′ and 5′-CTGCAGCCCGGGGGATCCG-3′ were annealed, phosphorylated and ligated into the *Eco* RI/*Sac* I sites of the pGEM3/88. A 1.9 kb *Bam* HI/*Pst* I fragment of the pBSII-EB2 plasmid was subcloned into the *Bam* HI/*Pst* I sites of the resultant plasmid to obtain pGEM3/1986. Finally, a 2 kb *Bam* HI/*Hin*dIII fragment of the pGEM3/1986 plasmid was subcloned into the *Bgl* II/*Hin*dIII sites of the pGL3-Basic vector to produce pmZHX1/luc1986. With combinations of upstream PCR primers, Luc405, 5′-ccggagatctTGAACGGGGCGGAGCCTCG-3′; Luc209, 5′-ccggagatctTCGAGTGGGGTGCCCGGC-3′; Luc59, 5′-ccggagatctGGCCCTCCGAGTGGTTCCG-3′; Luc40, 5′-ccggagatctTCCGGGGTTAGGGTTCACG-3′, and a common downstream primer LucR, 5′-CTTTATGTTTTTGGCGTCTTCC-3′, PCR reactions were carried out using the pmZHX1/luc1986 as a template. After digestion with *Bgl* II and *Hin*dIII, amplified DNA fragments were subcloned into the *Bgl* II/*Hin*dIII sites of the pGL3-Basic to obtain pmZHX1/luc405, pmZHX1/luc209, pmZHX1/luc59, and pmZHX1/luc44, respectively. Custom-synthesized sense and anti-sense oligonucleotides that contain the nucleotide sequence from −59 to +19 of the mouse *ZHX1* gene were annealed and phosphorylated using the T4 polynucleotide kinase reaction. The double-stranded DNA fragment was subcloned into the *Bgl* II/*Hin*dIII sites of the pGL3-Basic to produce pmZHX1/luc-59/ + 19.

Site-directed mutagenesis was carried out using the Quik-change site-directed mutagenesis kit. The following complementary oligonucleotides, mut1s, 5′-GATTCCGGAGATCTGAATTCCCGAGTGGTTCCGG-3′, and mut1as: 5′-CCGGAACCACTCGGGAATTCAGATCTCCGGAATC-3′, mut2s, 5′-GGAGATCTGGCCCTGAATTCGGTTCCGGGGTTAG-3′, and mut2as: 5′-CTAACCCCGGAACC

GAATTCAGGGCCAGATCTCC-3′, mutAs: 5′-GGCCCT CCGAGTGAATTCGGGGTTAGGGTT-3′, and mutAas: 5′-AACCCTAACCCCGAATTCACTCGGAGGGCC-3′, and, mutBs: 5′-CGCTCTGAAATGCAAGGAATTCGCG GCGAGGCGCTGAG-3′ and mutBas: 5′-CTCAGCGCCT CGCCGCGAATTCCTTGCATTTCAGAGCG-3′, were employed for the PCR-based mutagenesis, respectively (mutated bases are underlined). After confirmation of the nucleotide sequence, these *Bgl*II/*Hin*dIII fragments were subcloned into the *Bgl*II/*Hin*dIII sites of the pGL3-Basic to produce pmZHX1/luc59-mut1, pmZHX1/luc59-mut2, pmZHX1/luc59-mutA, and pmZHX1/luc 59-mutB, respectively. The pmZHX1/luc 59-mutAB was constructed using pmZHX1/luc 59-mutA as the template and mutBs and mutBas as the primers.

2.8. DNA transfections and luciferase assays

F9 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator. All plasmids used for transfection were prepared using a QIAGEN plasmid kit, followed by CsCl gradient ultracentrifugation. DNA transfections were carried out using the Lipofectamine Plus reagent as described previously (Shimada et al., 2001). Briefly, 5×10^4 cells per well were inoculated in a 24-well plate on the day prior to transfection. Three hundred nanograms of an indicated reporter plasmid and 2 ng of the pRL-CMV plasmid were used. After 3 h of transfection, the medium was changed. After 48 h, the cells were harvested and firefly and sea pansy luciferase activities were determined using a Berthold Lumat model LB 9501 luminometer (Wildbad, Germany). Firefly luciferase activities (relative light units) were normalized by sea pansy luciferase activities.

2.9. Electrophoretic mobility shift assays (EMSAs)

Preparation of nuclear extracts was carried out as described previously (Yamada et al., 1997). The nucleotide sequences of oligonucleotides used in EMSA are listed in Table 1. EMSAs were performed as described previously (Yamada et al., 2000). For a competition analysis, a 200-fold molar excess of competitor DNAs was added to the binding mixture. For supershift assays, antibodies were first mixed with the nuclear extracts on ice for 30 min and a ^{32}P-labeled probe was then added to the mixture, followed by a 30-min incubation. After completion of the binding, the mixture was subjected to electrophoresis on a 6% polyacrylamide gel (acrylamide/bis-acrylamide = 19 : 1) in 44.5 mM Tris–HCl (pH 8.0), 44.5 mM boric acid, and 1 mM EDTA at 200 V for 1 h. The gels were dried and exposed to a Fujix imaging plate (Kanagawa, Japan). Signals were detected with the Fujix BAS-2000 image analysing system.

3. Results

3.1. Alternative splicing forms of mouse ZHX1 mRNA

In order to explore cell line(s) that express endogenous mouse ZHX1 mRNA, we initially employed a RT–PCR method. Total RNA from mouse teratocarcinoma F9 cells was analysed. Although the predicted size of the product was 354 bp, two bands, corresponding to the products were unexpectedly detected (Fig. 1A). We isolated both bands and determined their nucleotide sequences. The nucleotide sequence of the small band was indeed identical to that of previously reported mouse ZHX1 cDNA (Barthelemy et al., 1996). In contrast, the 480 bp large band corresponded to the small band in which 126 bp nucleotide sequences had been inserted (Fig. 1B). To examine the issue of whether these ZHX1 mRNAs exhibit tissue-specific distribution, we performed RT–PCR with total RNA from various tissues. As shown in Fig. 1C, the two forms of mouse ZHX1 mRNA

Table 1
Nucleotide sequences of oligonucleotides used in EMSAs

Oligonucleotides	Nucleotide sequences
Box A	5′-gatcCCGAGTGGTTCCGGGGTTAG-3′ 3′-GGCTCACCAAGGCCCCAATCctag-5′
mut Box A	5′-gatcCCGAGTGAATTCGGGGTTAG-3′ 3′-GGCTCACTTAAGCCCCCAATCctag-5′
Box B	5′-gatcATGCAAGATGGCGGCGGCGA-3′ 3′-TACGTTCTACCGCCGCCGCTctag-5′
mut Box B	5′-gatcATGCAAGGAATTCGCGGCGA-3′ 3′-TACGTTCCTTAAGCGCCGCTctag-5′
PEA3	5′-GATCTCGAGCAGGAAGTTCGA-3′ 3′-CTAGAGCTCGTCCTTCAAGCT-5′
E2F-1	5′-ATTTAAGTTTCGCGCCCTTTCTCAA-3′ 3′-TAAATTCAAAGCGCGGGAAAGAGTT-5′
YY1	5′-CGCTCCGCGGCCATCTTGGCGGCTGGT-3′ 3′-GCGAGGCGCCGGTAGAACCGCCGACCA-5′
NF-E2	5′-TGGGGAACCTGTGCTGAGTCACTGGAG-3′ 3′-ACCCCTTGGACACGACTCAGTGACCTC-5′
Stat3	5′-GATCCTTCTGGGAATTCCTAGATC-3′ 3′-CTAGGAAGACCCTTAAGGATCTAG-5′
NF-1	5′-TTATCATATTGGCTTCAATCCAAAA-3′ 3′-AATAGTATAACCGAAGTTAGGTTTT-5′
AP-1	5′-CGCTTGATGACTCAGCCGGAA-3′ 3′-GCGAACTACTGAGTCGGCCTT-5′
AP-2	5′-GATCGAACTGACCGCCCGCGGCCCGT-3′ 3′-CTAGCTTGACTGGCGGGCGCCGGGCA-5′
C/EBP	5′-TGCAGATTGCGCAATCTGCA-3′ 3′-ACGTCTAACGCGTTAGACGT-5′
Oct-1	5′-TGTCGAATGCAAATCACTAGAA-3′ 3′-ACAGCTTACGTTTAGTGATCTT-5′
GATA	5′-CACTTGATAACAGAAAGTGATAACTCT-3′ 3′-GTGAACTATTGTCTTTCACTATTGAGA-5′
USF	5′-CCGGTAGGCCACGTGACCGGGT-3′ 3′-ATCCGGTGCACTGGCCCAGGCC-5′
Sp1	5′-ATTCGATCGGGGCGGGGCGAGC-3′ 3′-TAAGCTAGCCCCGCCCCGCTCG-5′
Egr-1	5′-GATCTCGAGCAGGAAGTTCGA-3′ 3′-CTAGAGCTCGTCCTTCAAGCT-5′

Mutated bases are underlined.

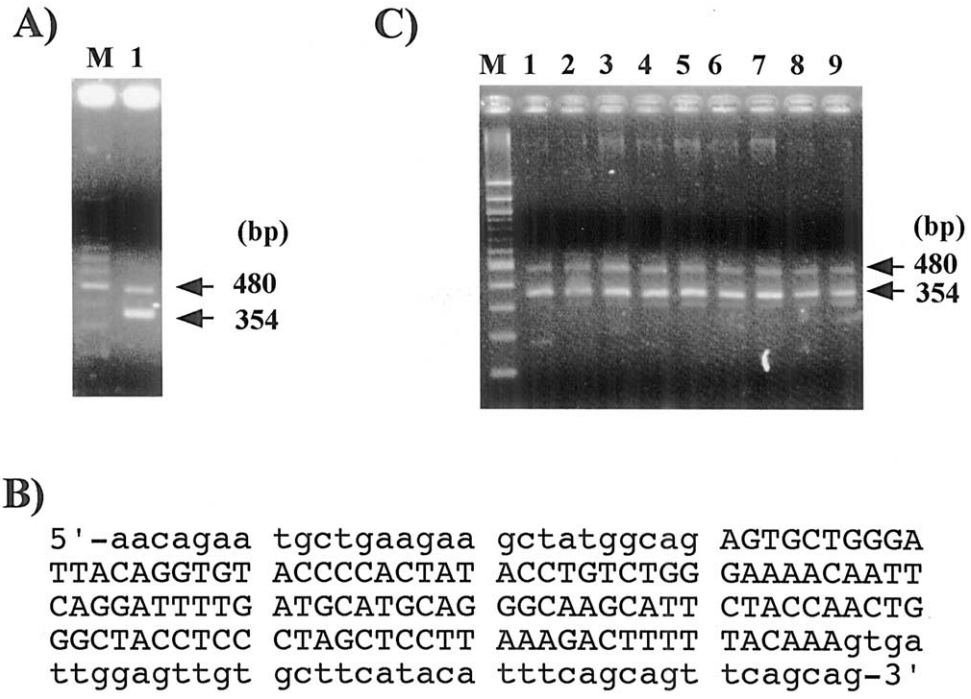

Fig. 1. RT–PCR analysis and the nucleotide sequence of a novel mouse ZHX1 mRNA. (A,C) Total RNAs (1 μg) were subjected to RT–PCR. The products were loaded on a 2% agarose gel, then visualized with an ethidium bromide staining. (A) M, DNA size marker; lane 1, F9 cells. (C) M, DNA size marker; lane 1, heart; lane 2, ovary; lane 3, testis; lane 4, brain; lane 5, kidney; lane 6, liver; lane 7, lung; lane 8, spleen; lane 9, F9 cells. (B) A part of the nucleotide sequence of the large band shown in (A). Reported and novel nucleotide sequences are shown in lowercase and uppercase, respectively.

were expressed in all tissues. The ratio of intensity between the large and small bands was constant. In order to determine the issue of whether these two ZHX1 mRNAs are produced from a single gene or two different genes, we next carried out a genomic Southern blot analysis. Only a single band was detected in the 129 mouse genomic DNA, after digestion with *Eco* RI, *Bam* HI, or *Hin*dIII (Fig. 2). These results indicate that a single copy of the *ZHX1* gene per haploid mouse genome is present and that the two forms of mouse ZHX1 mRNAs may be ubiquitously produced from a single gene by alternative splicing.

3.2. Structure of the mouse ZHX1 gene

We then screened 129 mouse spleen genomic libraries to obtain some genomic clones of the mouse *ZHX1* gene. When 5×10^5 independent clones were screened, seven distinct clones were isolated. We determined the complete genomic nucleotide sequence of the mouse *ZHX1* gene (The nucleotide sequence of the mouse *ZHX1* gene was submitted to the DNA Data Bank of Japan accession no. AB078421). The mouse *ZHX1* gene spans 28,885 bp and consists of five exons and four introns (Fig. 3). Nucleotide sequence inserted in the large band of the RT–PCR products corresponded to the third exon. Exons 1–3 and exon 5 encoded 5′- and 3′-noncoding sequence, respectively. Surprisingly, exon 4 encoded a part of the 5′-noncoding sequence, an entire coding sequence, and a part of the 3′-noncoding sequence. The exon–intron boundaries are listed in Table 2. GT and AG residues are present at the 5′ and 3′ boundaries of introns, consistent with the consensus sequence for the splicing of eukaryotic mRNA (Sharp, 1981; Mount, 1982). These results indicate that two species of ZHX1 mRNA with or without the nucleotide sequence of the third exon are produced by an alternative splicing.

3.3. Determination of the transcription initiation site

We determined the transcription initiation site of the mouse *ZHX1* gene by nuclease S1 mapping. As shown in Fig. 4, multiple specific bands were observed in mouse liver and lung total RNAs but not in yeast tRNA. Same patterns were obtained in three independent experiments. As the size of the major band corresponded to 50 bases, the G residue was numbered as position +1. The other two minor transcription initiation sites were nucleotides A and C, which are located at positions +3 and +6, respectively.

3.4. Identification of transcriptional regulatory region of the mouse ZHX1 gene

To investigate the transcriptional regulatory region of the mouse *ZHX1* gene, we constructed the pmZHX1/luc1986, in which a fragment from nucleotide −1986 to +50 relative to the major transcription initiation site of the mouse *ZHX1* gene was linked to the upstream of the firefly luciferase reporter gene. The pGL3-Basic, a promoter-less luciferase reporter vector, and pmZHX1/luc1986 were transiently

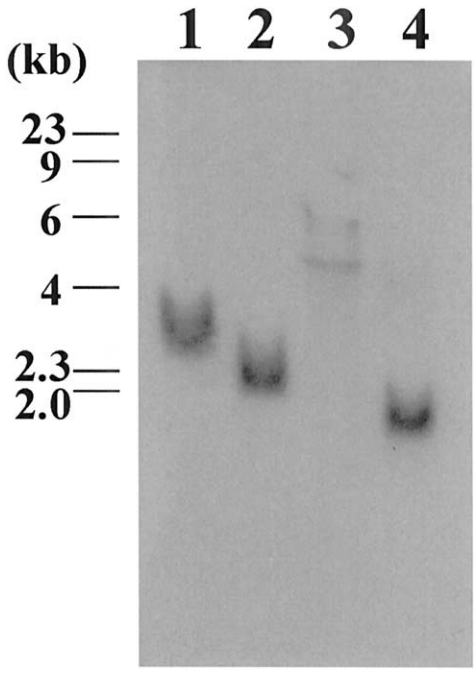

Fig. 2. Southern blot analysis of genomic DNA. The 129 mouse genomic DNA (10 μg) was digested with various restriction endonucleases. The reactions were subjected to a 0.7% agarose gel electrophoresis, transferred to a Hybond N filter, and hybridized with the ^{32}P-labeled probe 2. Size of DNA is depicted on the left. Lane 1, *Eco* RI; lane 2, *Bam* HI; lane 3, *Pst* I; lane 4, *Hin*dIII.

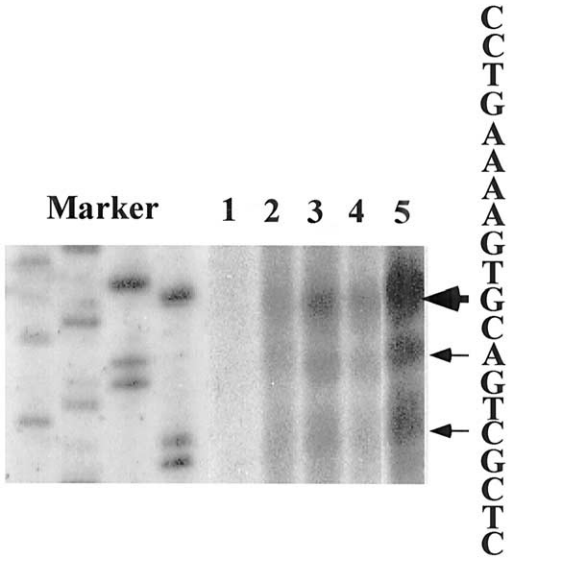

Fig. 4. Determination of the transcription initiation site by nuclease S1 analysis. Various amounts of total RNAs from mouse liver and lung and yeast tRNA were hybridized with the ^{32}P-labeled DNA probe, and then digested with nuclease S1. After denaturation, the products were electrophoresed on a 7% polyacrylamide/7 M urea gel along with sequence ladders of M13 mp18 DNA as size markers. Lane 1, 50 μg yeast tRNA; lanes 2 and 3, 10 and 50 μg of mouse liver total RNA; lanes 4 and 5, 10 and 50 μg of mouse lung total RNA. A part of the nucleotide sequence of the mouse *ZHX1* gene is shown on the right and transcription initiation sites are indicated by arrows.

transfected into F9 cells using a lipofection method and their luciferase activities determined. As shown in Fig. 5, when the pGL3-Basic was transfected, the luciferase activity was quite low. In contrast, the pmZHX1/luc1986 showed a 30-fold higher luciferase activity than that of the pGL3-Basic. The effects of successive 5′-deletion mutants of the mouse *ZHX1* gene promoter fused to the luciferase reporter plasmid on luciferase activity were next evaluated (Fig. 5). While the deletion up to −803 (pmZHX1/luc803) caused only a marginal effect, deletion up to −405 (pmZHX1/luc405) resulted in almost a 4-fold higher value than that of the pmZHX1/luc1986. Luciferase activities remained unchanged in the deletion constructs, such as the pmZHX1/luc209, pmZHX1/luc88, and pmZHX1/luc59. In contrast, the luciferase activity of a construct deleted up to −44 (pmZHX1/luc44) dramatically decreased to 26.1% of that of the pmZHX1/luc1986. These results indicate that an inhibitory region exists from −803 to −406 and that the nucleotide sequence between −59 and +50 is required for the full promoter activity of the mouse *ZHX1* gene. To identify the 3′-end point of the mouse *ZHX1* gene promoter, we prepared a 3′-deletion construct. When a DNA fragment that contains −59 to +19 was inserted into the luciferase reporter plasmid (pmZHX1/luc-59/ + 19), the luciferase activity decreased to 24.0% of that of the pmZHX1/luc1986.

These results indicate that at least two positive regions, nucleotide sequence between −59 and −45 and between +20 and +50 are contributed to full promoter activity of the mouse *ZHX1* gene.

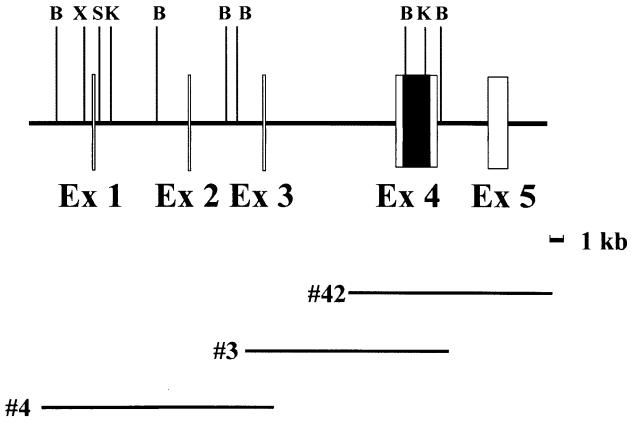

Fig. 3. Structure of the mouse *ZHX1* gene. Exons are depicted by boxes. Open and hatched boxes represent noncoding and coding sequences, respectively. The scale for 1 kb is indicated. Some restriction endonuclease sites are indicated as follows: B, *Bam* HI; K, *Kpn* I; S, *Sma* I; X, *Xho* I. Some clones, #3, #4, and #42 are depicted by bars at the bottom.

3.5. Identification of cis-acting elements of the mouse ZHX1 gene

The nucleotide sequence of the promoter region of mouse *ZHX1* gene is shown in Fig. 6. These sequences were submitted to the TFSEARCH program in a search for putative transcription factor-binding sites. While a putative binding sequence was not found in the nucleotide sequence

Table 2
Nucleotide sequences of exon–intron boundaries of the mouse *ZHX1* gene

Exon no.	Exon size (bp)	Exon/intron junction		Intron size (kb)
		5′ Donor/	3′ Acceptor	
1	61	CCCCTGCAG gtatct/	ttttttttgtag GAGACTGCT	6.7
2	111	CTATGGCAG gtatgt/	tctacttccag AGTGCTGGG	5.1
3	126	TTTTACAAA gtaagt/	ttccaatgtag GTGATTGGA	8.9
4	2859	GACTGAAAT gtaagt/	ttctttttatag CTACTTAAA	3.4
5	1481			
Consensus sequence		CAG gtaagt/ Ag	ttttttttncag G ccccccc t	

The sizes of the exons and introns, along with the exon–intron boundary sequences, are shown. Exon sequences are given in capitals and introns in lower case letters. Each intron begins with a GT and ends with an AG. The consensus sequence was obtained from Sharp (1981); Mount (1982).

between −-59 and −45, a putative binding sequence of the GATA family, 5′-AGATG-3′, was found between +20 and +24. We then attempted to identify bona fide *cis*-acting elements by site-directed mutagenesis analysis (Fig. 7A). The nucleotide sequence of 5′-GAATTC-3′ were differentially introduced into the upstream regulatory regions of the mouse *ZHX1* gene promoter. When the block mutation was introduced at the −59/−54 (pmZHX1/luc59-mut1) or −53/−48 (pmZHX1/luc59-mut2) sites of the pmZHX1/luc59 plasmid, luciferase activities were not affected (data not shown). However, when the mutation was introduced at −47/−42 site (pmZHX1/luc59-mutA), the luciferase activity was decreased to 3.1% of that of wild-type pmZHX1/luc59 (Fig. 7B). The same block mutation was introduced at the +22/+27 site (pmZHX1/luc59-mutB) of the pmZHX1/luc59 plasmid, which is overlapped with a putative GATA-binding sequence. The luciferase activity was also decreased to 17.5% of that of wild-type pmZHX1/luc59. When both elements were mutated (pmZHX1/luc59-mutAB), promoter activity was totally abolished. We named the upstream and downstream elements Box A and Box B, respectively.

Thus, these results indicate that the promoter of the mouse *ZHX1* gene consists of at least two *cis*-acting elements that are located between −47 and −42, and +22 and +27, respectively and that they synergistically activate transcription of the mouse *ZHX1* gene.

3.6. PEA3 binds to Box A in vitro

We carried out EMSAs to identify proteins in the nuclear extracts of F9 cells which interact with these boxes. No consensus binding sequence of transcription factors were found in Box A. Therefore, we used the following oligonucleotides for the binding sequence of ubiquitously or broadly expressed transcription factors for competition experiments; PEA3, a member of the Ets family, E2F-1, Yin and Yang 1 (YY1), nuclear factor- erythroid-2 (NF-E2), signal transducers and activators of transcription (Stat) 3, nuclear factor-1 (NF-1), activator protein-1 (AP-1), activator protein-2 (AP-2), CCAAT/Enhancer-binding protein

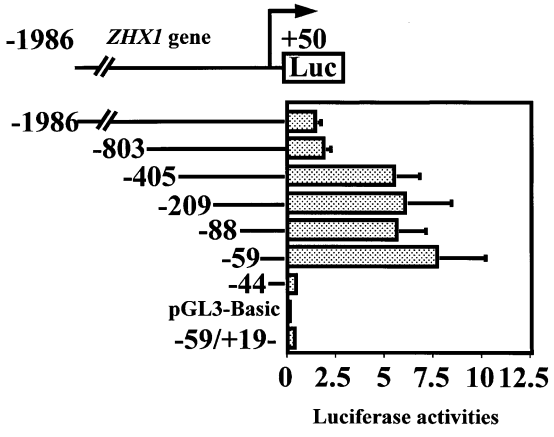

Fig. 5. Deletion analysis of promoter activity of the mouse *ZHX1* gene. Schematic diagrams of the 5′- or 3′-deletion reporter constructs are depicted on the left. F9 cells were transiently transfected with 300 ng of luciferase reporter plasmids along with 2 ng of the pRL-CMV. Firefly luciferase activities normalized by sea pansy luciferase activities are shown on the right. All values shown represent the mean ± standard error of at least three independent experiments.

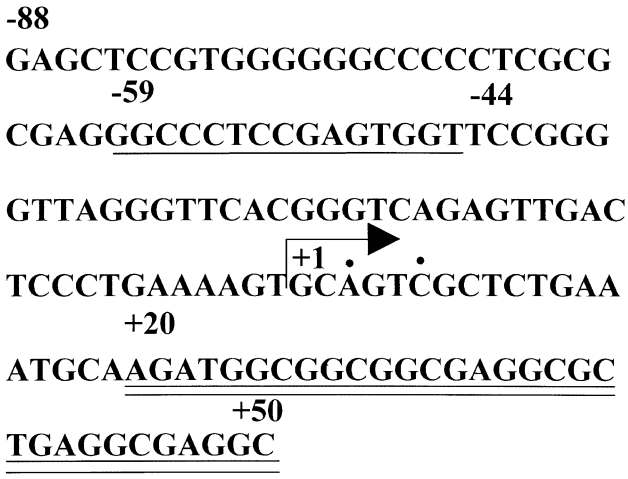

Fig. 6. Nucleotide sequence of the promoter region and a part of the first exon of the mouse *ZHX1* gene. A major transcription initiation site is numbered as +1 and indicated by an arrow. Other minor transcription initiation sites are indicated by dots. Upstream and downstream regulatory regions are underlined and double-underlined, respectively.

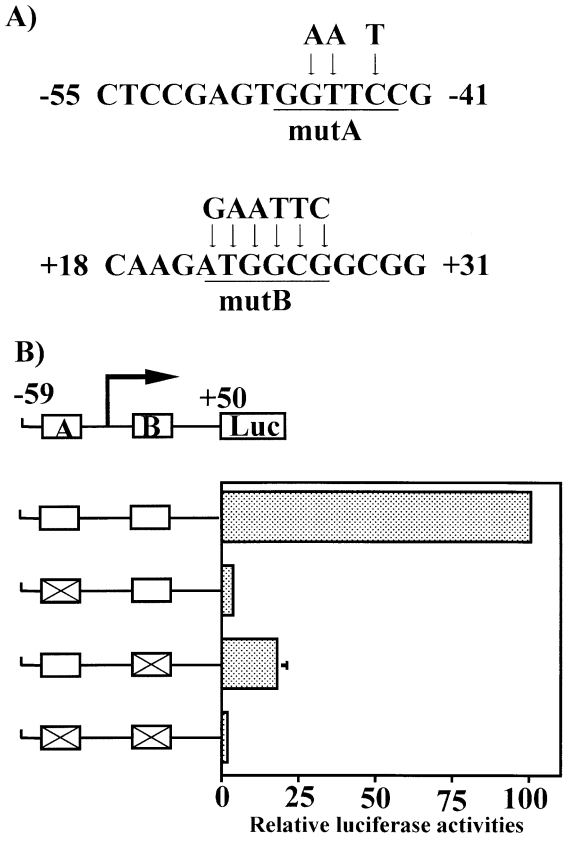

Fig. 7. Determination of *cis*-acting elements of the mouse *ZHX1* gene promoter by site-directed mutagenesis. (A) Nucleotide sequence of mutations of regulatory regions of the mouse *ZHX1* gene. (B) Effects the mutations on promoter activity. Wild-type, either or combinations of mutation of Boxes A and B of the *ZHX1* gene promoter, were linked to the luciferase plasmid. The value of the wild-type pmZHX1/luc59 was defined as 100. Each value represents the mean ± standard error of at least three independent experiments.

(C/EBP), octamer-binding protein-1 (Oct-1), GATA-binding protein (GATA), upstream stimulatory factor (USF), specificity protein 1 (Sp1), and early growth response gene-1 (Egr-1) as well as wild-type and mutated Box A oligonucleotides of the mouse *ZHX1* gene.

When the ^{32}P-labeled Box A oligonucleotide was mixed with nuclear extracts of F9 cells, DNA-protein binding complexes were observed (Fig. 8A). A complex disappeared on the addition of a 200-fold molar excess of unlabeled Box A oligonucleotide to the binding mixture but not the mut Box A oligonucleotide (Fig. 8A). These results indicate that a protein specifically binds to Box A. The addition of a 200-fold molar excess of oligonucleotides for E2F-1-, YY1-, NF-E2-, Stat 3-, NF-1-, AP-1-, AP-2-, C/EBP-, Oct-1-, GATA-, USF-, and the Sp1-binding sequence resulted in no competition. Interestingly, the addition of a 200-fold molar excess of unlabeled PEA3-binding sequence led to the disappearance of the binding complex.

We then examined the issue of whether PEA3 is capable of binding to Box A using a specific antiserum against PEA3. As shown in Fig. 8B, the incubation of nuclear extracts of F9 cells with anti-PEA3 antibody resulted in a decrease in the abundance of the band. In contrast, a specific band was not affected with anti-Ets1/Ets2 antiserum. These results indicate that PEA3 binds to Box A.

3.7. YY1 binds to Box B in vitro

Box B contains a binding sequence for the GATA family. As shown in Fig. 9A, mixing the ^{32}P-labeled Box B oligonucleotide with nuclear extracts of F9 cells resulted in the formation of DNA-protein complexes. These complexes disappeared on the addition of a 200-fold molar excess of unlabeled Box B oligonucleotide. In contrast, the formation of these complexes was not affected by the addition of the mut Box B oligonucleotide. Surprisingly, the addition of the GATA-binding sequence had no effect on these specific DNA-protein complexes. These results suggest that Box B-binding proteins are not members of the GATA family. We also used other binding sequences of transcription factor in competition analyses. Only the addition of the YY1-binding sequence to the binding mixture caused the disappearance of these complexes. This result indicates that YY1 or a closely related protein recognizes and binds to Box B.

We then examined the binding of YY1 to Box B using a supershift assay. When the Box B oligonucleotide was used as probe, supershifted bands and a parallel decrease in the intensity of the DNA-protein complex bands were detected on incubation with anti-YY1 antiserum (Fig. 9B). In contrast, no super-shifted band was formed with anti-Ets1/Ets2 antiserum. This result indicates that YY1 binds to Box B.

4. Discussion

The mouse *ZHX1* gene was cloned and its transcriptional regulatory mechanism in F9 cells characterized. This is the first demonstration of the cloning and characterization of all the *ZHX1* genes. In the process of searching the cell lines which express endogenous mouse ZHX1 mRNA for promoter analysis, two different transcripts were unexpectedly observed (Fig. 1). The same transcripts were observed in various tissues (Fig. 1). Genomic Southern blot analysis showed that the mouse *ZHX1* gene exists as a single gene per haploid genome (Fig. 2). In addition, molecular cloning and a determination of the nucleotide sequence of the mouse *ZHX1* gene revealed that these transcripts are produced from a single gene by an alternative splicing of exon 3 (Fig. 3). Alternative splicing sometimes leads to the production of two or more closely related but distinct proteins from a single gene, thus playing an important role in cell- or tissue-specific expression or in the stage-specific expression of the gene (Yamada and Noguchi, 1999). However, in the case of the mouse *ZHX1* gene, the protein structure is not affected by the alternative splicing since the exon 3 encodes the 5′-

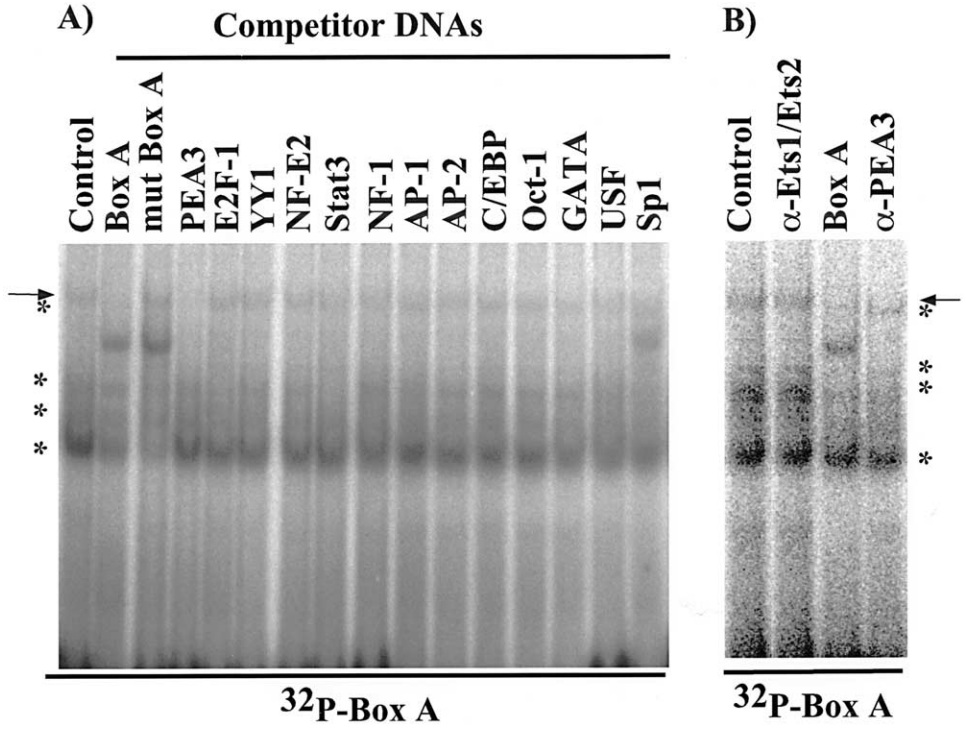

Fig. 8. PEA3 binds to Box A. (A) A ^{32}P-labeled Box A was incubated with 10 μg of nuclear extracts of F9 cells. The competitor DNAs shown at the top were used at a 200-fold molar excess. An arrow on the left indicates the position of a specific protein/DNA complex. Asterisks indicate non-specific binding complexes. (B) The nuclear extracts were preincubated with antiserum directed against Ets1/Ets2 or PEA3 for 30 min prior to the addition of the probe. Antiserum is shown at the top. The arrow on the right indicate PEA3/DNA complex. Asterisks indicate non-specific binding complexes. The protein/DNA complex was separated by a 6% PAGE and subjected to BAS-2000 image analysing system.

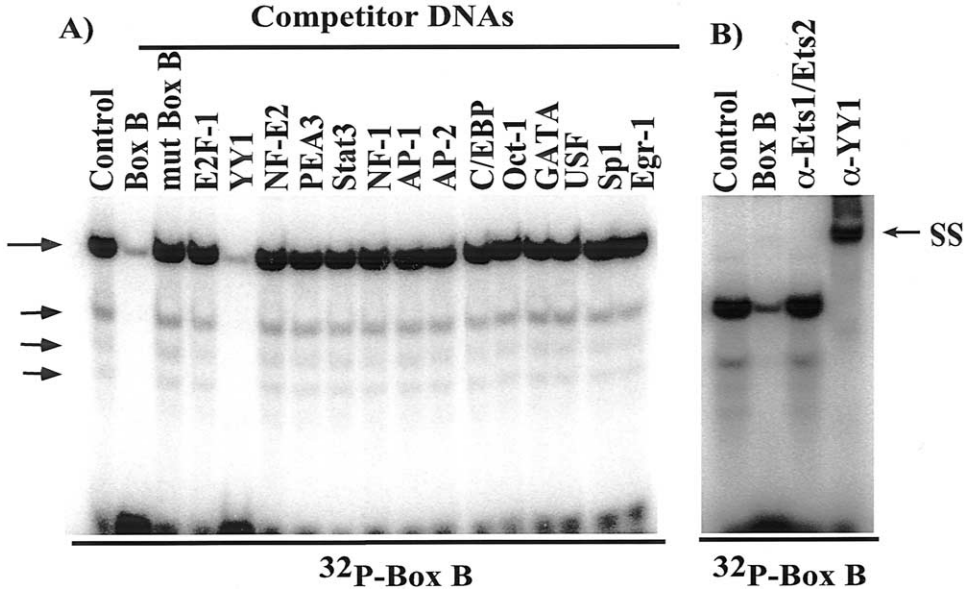

Fig. 9. YY1 binds to Box B. (A) A ^{32}P-labeled Box B was incubated with 10 μg of nuclear extracts of F9 cells. The competitor DNAs shown at the top were used at a 200-fold molar excess. The arrows on the left indicate the positions of the specific protein/DNA complexes. (B) The nuclear extracts were preincubated with antiserum directed against Ets1/Ets2 or YY1 for 30 min prior to the addition of the probe. Antiserum is shown at the top. The arrows on the right indicate a YY1/DNA complex-supershifted complex (SS) with antibody. Protein/DNA complexes were separated by a 6% PAGE and subjected to BAS-2000 image analysing system.

noncoding region. Thus, the biological role of an alternative splicing of the mouse *ZHX1* gene is unclear at present. The mouse *ZHX1* gene is approximately 29 kb in size and consists of five exons and four introns (Fig. 3). Interestingly, the fourth exon, 2.9 kb in size, encodes a part of the 5′-noncoding sequence, an entire coding sequence, and a part of the 3′-noncoding sequence. It has been reported that genes encoding members of the CCAAT/Enhancer-binding protein family are intronless (Landschulz et al., 1988; Akira et al., 1990; Descombes et al., 1990). However, it is rare that a single exon encodes an entire open reading frame and other exons encode the noncoding sequences.

Nuclease S1 mapping demonstrated that the transcription initiation site of the mouse *ZHX1* gene is multiple (Fig. 4). This result can be explained by the lack of a TATA box in the upstream region of the gene. It has been well documented that the TATA box is essential for an accurate transcription initiation site, and that the transcription initiation site is variable in most TATA-less genes (de Launoit et al., 1997; Igarashi et al., 1997).

Successive deletion analysis indicates that a region of the nucleotide sequence from −59 to +50 is required for full promoter activity (Fig. 5). The region consists of at least two positive *cis*-acting elements, which are located in nucleotide sequences from −59 to −45 and +20 to +50, respectively (Fig. 6). Site-directed mutagenesis revealed that both Box A (5′-GGTTCC-3′) and Box B (5′-ATGGCG-3′) within or overlapping with above elements are essential for promoter activity (Fig. 7). The Box B exists just downstream of the transcription initiation sites of the mouse *ZHX1* gene. Such an element is referred to as the ‘initiator’ and is localized in TATA-less genes. In addition to these positive regulatory elements, negative regulatory element(s) also exist in the nucleotide sequence between −803 and −406 (Fig. 5). Further studies will be required for the characterization of this region.

Competition and supershift analysis in the EMSAs showed that PEA3 binds to Box A (Fig. 8). PEA3 belongs to the Ets family. It has been reported that the Ets family consists of several members, such as Ets-1, Ets-2, PU.1, PEA3, Elf-1, Elk-1, Erg-1, Erg-2, Fli-1, etc. (Martin et al., 1992; Li et al., 2000; Yordy and Muise-Helmericks, 2000). They share a highly conserved DNA-binding domain, and recognize similar nucleotide sequences that share a centrally located 5′-GGAA-3′ element. An inverted element, 5′-TTCC-3′, exists nucleotide sequence between −45 and −42 of the Box A (see Fig. 6). The introduction of a mutation of the element causes a decrease in promoter activity and a concomitant reduction in binding activity of a DNA-binding protein (Figs. 7 and 8). It has been reported that Ets2 and PEA3 but not Ets1 is expressed in F9 cells (Martin et al., 1992; Kola et al., 1993). However, the protein binding pattern to Box A in the EMSAs remained unchanged even after the addition of two types of anti-Ets2 antiserum (Fig. 8 and data not shown). Therefore, we conclude that Ets2 is not contained in the Box A-protein binding complex. Since the consensus binding sequence of PEA3 and Ets2 is slightly different from the outer sequence of the 5′-GGAA-3′, it is understandable that only PEA3 binds to Box A. The Ets transcription factors are involved in tumorigenesis and developmental processes (de Launoit et al., 2000). In addition, PEA3 is activated through both the Ras-dependent and other kinase pathways. Although their target genes are multiple, they are mainly involved in organogenesis and metastatic process, especially in the case of breast cancer (de Launoit et al., 1997).

YY1 (also referred to as the nuclear factor-E1 or upstream conserved region binding protein) binds to Box B in the EMSAs (Fig. 9). A mutation that interferes with the binding of YY1 to the Box B led to a decrease of promoter activity (Figs. 7 and 9). The nucleotide sequence (5′-AAGATGGCG-3′) of the YY1-binding site of Box B is perfectly matched with that of the mouse DNA topoisomerase IIIα gene (Park et al., 2001). YY1 ubiquitously expresses and positively or negatively regulates the transcription of several cellular genes (Park and Atchison, 1991; Flanagan et al., 1992; Thomas and Seto, 1999). It has been reported that YY1 is a 65 kDa member of the GLI-Krüppel family of zinc-finger transcription factors and that it is one of initiator-binding proteins (Thomas and Seto, 1999). The precise mechanism of its dual effect in transcriptional regulation has not yet been elucidated (Thomas and Seto, 1999). A possible explanation is that the promoter context and factors already present at a promoter dictate which function of YY1 is displayed at that promoter. Alternatively, it is conceivable that the dual nature depends on the different pre-existing YY1-cofactor complexes being recruited to a promoter under different conditions (Thomas and Seto, 1999). Indeed, it has been reported that YY1 interacts with other transcription factors including the cyclic AMP response element-binding protein-binding protein (CBP) co-activator or the histone deacetylase 1 repressor (Austen et al., 1997; Thomas and Seto, 1999; Coull et al., 2000).

Both PEA3- and YY1-binding sites were also localized in the polyomavirus enhancer A (Shivakumar and Das, 1998). A reporter gene analysis revealed that the activity of enhancer A is determined by a combinatorial effect of these proteins (Shivakumar and Das, 1998). In addition, the putative interaction of PEA3 and YY1 is also found in the serum response element in mouse embryos (Liu et al., 1995). It has been reported that both PEA3 and YY1 are expressed in F9 cells (Martin et al., 1992; Satyamaoorthy et al., 1993). Our results clearly show that PEA3 and YY1 in nuclear extracts of F9 cells specifically bind to Box A and Box B, respectively. Therefore, it is possible that these two universal transcription factors directly or indirectly interact with each other and synergistically control the transcriptional regulation of the mouse *ZHX1* gene in F9 cells.

It has been reported that IL-2 induces the expression of mouse ZHX1 mRNA in a T cell line, 18.111 (Herblot et al., 1999). Key molecules involved in the IL-2 signaling

pathway are Janus kinase (JAK) 1, JAK3 and members of the Stats transcription factor family (Herblot et al., 1999). The issue of whether IL-2 up-regulates the expression of the ZHX1 mRNA via these molecules is presently unclear. In contrast, one clinical research report on the evaluation of the expression and DNA binding activity of YY1 in peripheral blood mononuclear cell of HIV-infected individuals before, during and after the administration of IL-2 in association with antiretroviral therapy, found that YY1 was profoundly down-modulated during the administration of IL-2 (Bovolenta et al., 1999). If IL-2 exerts the same effect in T cells, YY1 may function as a repressor, in contrast to its putative role in F9 cells. In this case, other mechanisms may be required for the transcriptional regulation of the mouse *ZHX1* gene.

The issue of whether PEA3 and YY1 actually stimulates transcription from the mouse *ZHX1* gene promoter in vivo, whether PEA3 physically interacts with YY1, whether a co-activator(s) such as p300/CBP exhibiting histone acetyltransferase activity is involved in the transcriptional regulation of the *ZHX1* gene, and how IL-2 stimulates *ZHX1* gene expression remains to be determined. Further studies will be required to address these questions.

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