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ZHX2 and ZHX3 repress cancer markers in normal hepatocytes

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1. ABSTRACT

ZHX2 and ZHX3 are the members of the ZHX transcriptional repressor family. To investigate the regulatory role of the repressors in hepatocytes and their involvement in carcinogenesis, the expression levels of ZHX2 and ZHX3 mRNAs were examined. The dRLh-84 hepatoma cells considerably expressed cancer marker genes PKM and HK II that are expressed in developing fetal tissues and cancer cells but repressed in normal hepatocytes. In dRLh-84 cells, the expression levels of ZHX2 and ZHX3 were very low compared with rat hepatocytes. Upon the reporter gene analysis utilizing the promoter region of these genes, ZHX3 repressed the transcription of the reporter luciferase gene from both promoters while ZHX2 only repressed that from HK II promoter. The promoter activity of alpha-fetoprotein was also repressed by the expression of ZHX2 in HLE hepatoma cells in a dose-dependent manner. We concluded that ZHX2 and ZHX3 were involved in the transcriptional repression of the hepatocellular cacinoma markers in normal hepatocytes, suggesting that the failure of the ZHX2 and/or ZHX3 expression might be a critical factor in the hepatocellular carcinogenesis.

2. INTRODUCTION

The ZHX transcriptional repressor family consists of three members, ZHX1, ZHX2, and ZHX3. All ZHX proteins structurally contain two Cys_2-His_2-type zinc-finger motifs responsible for DNA-binding and multiple homeodomains responsible for dimer-formation (1-5). The structure allows ZHX members to form homo- and hetero-dimers among the family members and bind the *cis*-DNA regulatory elements for transcriptional repression of genes (3, 4, 6-8). ZHX1 was isolated as an interacting protein with a transcription factor, nuclear factor-Y (NF-Y) that binds the Y-box (CCAAT) and induces transcription of genes involved in cellular proliferation and cancer development, such as M_2-type pyruvate kinase (PKM) and hexokinase II (HK II). PKM and HK II are expressed in fetal tissues and hepatoma cells but not normal adult hepatocytes, and the expression of PKM and HK II is positively regulated by NF-Y (9-13). Using a mammalian one-hybrid system, we reported the repressor function of ZHX1 (7). Therefore, the negative regulation of NF-Y-dependent transcription by ZHX1 may play an important role in the development of hepatocellular carcinoma. ZHX2 and ZHX3 isolated as ZHX1-binding proteins were

also transcriptional repressors involved in the negative regulation of genes associated with cellular proliferation as well as cancer development (2, 4). Upon analyses of 221 myeloma case, Harousseau *et al.* reported that a negative correlation between *ZHX2* gene expression and the expression of 30 proliferation-associated genes including genes that are positively regulated by NF-Y, and suggested that the loss of ZHX2 expression may up-regulates HOXB4 and confer myeloma cells a stem cell-like phenotype resulting in a resistance to chemotherapy (14). Therefore, ZHX family proteins may regulate the expression of proliferation genes including those regulated by NF-Y in various tissues as well as in cancer cells. In the present study, we investigated the role of members of the ZHX family in the expression of hepatocellular carcinoma biomarker genes, PKM, HKII, CDC25C (phosphatase involved in the mammalian cell cycle), and alpha-fetoprotein that are repressed in the normal hepatocytes.

3. MATERIALS AND METHODS

3.1. Materials

Collagenase was purchased from Yakult (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma Chemical Co. (Saint Louis, MO). The Schneider's medium, Trizol reagent, Superscript III, and Lipofectamine PLUS reagent were purchased from Invitrogen (Groningen, Netherlands). Alpha-^{32}P dCTP (110 TBq/ mmol) was purchased from GE Biosciences (Cleveland, OH). The Klenow fragment, BcaBest DNA labeling kit, ExTaq DNA polymerase, and restriction endonucleases were obtained from TaKaRa BIOMEDICALS (Kyoto, Japan). The Oligotex dT-30 super and GenoPure plasmid maxi kit were obtained from Roche Applied Science (Indianapolis, IN). The Biodyne membrane was obtained from PALL (ICN Biomedicals, Inc., Glen Cove, NY). The ExpressHyb hybridization solution, rat genomic DNA, yeast two-hybrid system 2, and X-alpha-gal were purchased from Clontech (Palo Alto, CA). The pGL3-Basic, dual luciferase reporter assay system, and pRL-CMV were obtained from Promega (Madison, WI). The pCMV-Tag2B plasmid was obtained from Stratagene (La Jolla, CA). The Big Dye terminator FS cycle sequencing kit was purchased from Applied Biosystems Japan (Tokyo, Japan).

3.2. Cells and cell culture

Rat MH_1C_1 hepatoma cells were purchased from the American Type Cell Collection (Manassas, VA). Rat H4IIE hepatoma cells were a generous gift from Dr. Daryl K. Granner (Vanderbilt University, U.S.A.). Rat dRLh-84 hepatoma cells and human HLE hepatoma cells were provided by the Japan Cancer Research Resources Bank (15, 16). Hepatocytes were freshly isolated from a male Sprague-Dawley rat liver (6 weeks of age, 170-190 g body weight) using a collagenase perfusion method (17). Schneider line 2 (SL2) cells, a *Drosophila* cell line, were a gift from Dr. Tamio Noguchi (Osaka Ohtani University, Tondabayashi, Japan).

MH_1C_1, H4IIE, dRLh-84, and HLE cells were grown in DMEM supplemented with 10 % fetal bovine serum and antibiotics at 37 ˚C in a 5 % CO_2 incubator. SL2 cells were grown in Schneider's medium supplemented with 10 % fetal bovine serum and antibiotics at 25 ˚C.

3.3. Probe DNAs

Oligonucleotides, MPK, 5'-ATTGCCCGAGAGGCAGAGGCTGCCATCTACCA-3', and MPKas, 5'-TGGTAGATGGCA-3', and HKII, 5'-ATCCGGGAGGCTGGGCAGAGATAGAAGCTTGGG-3', and HKIIas, 5'-CCCAAGCTTCTA-3', were annealed to form a double-stranded oligonucleotide, respectively, after which, it was labeled with the alpha-^{32}P-dCTP by Klenow reaction (18, 19). The probe for ZHX1, ZHX2, ZHX3, and 36B4 have been described previously (2, 4, 6, 20). ZHX1, ZHX2, ZHX3, and 36B4 cDNA were labeled with alpha-^{32}P-dCTP using the BcaBest DNA labeling kit.

3.4. Northern blot analysis

Poly A^+-RNA was prepared from various cells using the Trizol reagent and Oligotex dT-30 super. Poly A^+-RNA (5 micrograms/ lane) was subjected to denaturating agarose gel electrophoresis and then transferred to a Biodyne membrane. The filter was prehybridized in ExpressHyb solution at 68 ˚C for 30 min, and then hybridized with a ^{32}P-labeled probe and 20 micrograms/ ml heat-denatured herring testis DNA for 1 h. After washing at 50 ˚C in 0.1 x SSC, 0.1 % SDS, the filter was exposed to a FUJIX imaging plate (Kanagawa, Japan). Hybridization signals were detected with the FUJIX BAS-2000 image analyzing system.

3.5. Reverse transcription (RT)-polymerase chain reaction (PCR) analysis

Total RNA was isolated from various cells using the Trizol reagent. RT-PCR was performed as described previously (21). For ZHX1, 5'-CCTACACACATTTCCACAGG-3' and 5'-GGTTTCATCACTGGAGTCTAT-3' oligonucleotides, for ZHX2, 5'-GCCCGCCTGGTGACAGACAC-3' and 5'-CGCTGGGTGGCAAACCAGATTC-3' oligonucleotides, for ZHX3, 5'-GGAAAAAGATGTTCAATACAGTCA-3' and 5'-CACCTCTGGCACAGAGTCAAT-3' oligonucleotides, and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-GAACGGGAAGCTCACTGGCA-3' and 5'-TCCACCACCCTGTTGCTGTA-3' oligonucleotides, were used for RT-PCR primers, respectively. The PCR was conducted at 90 ˚ C for 3 min, followed by 27 (for ZHX1 and ZHX2), 31 cycles (for ZHX3), or 22 cycles (for GAPDH) at 90 ˚ C for 30 sec, 56 ˚ C for 30 sec and 72 ˚ C for 30 sec, and then at 72 ˚ C for 5 min. The number of cycles used for each gene was within the exponential part of the amplification curve. The reaction mixture was subjected to a 2 % agarose gel electrophoresis, then visualized with ethidium bromide-staining.

3.6. Plasmid construction

The pPac and pPac-Sp1 were generously provided by Dr. Guntram Suske (Philipps-Universität Marburg, Germany) (22, 23). The pPac-betaGal, pPacNF-YA, pPacNF-YB, and pPacNF-YC plasmids were generous

gifts from Dr. Timothy F. Osborne (University of California, Irvine) (24). The pGFP-ZHX1 (1-873), pGFP-ZHX3 (1-956), pPac-ZHX2, pCDC25C/Luc, pMPK287/Luc, and pZHX1-83/Luc were previously described (2, 4, 7, 12, 21). The pPac-Sp1 was digested with both *Xho*I and *Bam*HI and a large portion of the insert was removed. Oligonucleotides 5'-GATCTCGAGGGATCCG-3' and 5'-TCGACGGATCCCTCGA-3' were annealed, phosphorylated, and subcloned into the *Xho*I/ *Bam*HI sites of the resulting plasmid to obtain the pPac-XB. Each *Xho*I/*Bam*HI fragment from the pGFP-ZHX1 (1-873) and pGFP-ZHX3 (1-956) plasmids which contain full-length of human ZHX1 and ZHX3, respectively, was subcloned into the *Xho*I/*Bam*HI sites of the pPac-XB to produce pPac-ZHX1 and pPac-ZHX3, respectively. Genomic PCR was carried out using the rat genomic DNA as a template, and 5'-CCGGGGTACCGGGCTCTTCTCAGCCTCTATAAACC-3' and 5'-CCGGAAGCTTGATCCGTAAGGCTCAACTTCGCAA -3', as primers. After digestion with *Kpn*I and *Hind*III, amplified DNA fragments were subcloned into the *Kpn*I/ *Hind*III sites of the pGL3-Basic to obtain prHKII-157/Luc.

Mouse tail DNA was a kind gift from Dr. Yoshifumi Yokota (University of Fukui). With combinations of upstream PCR primers, S-mAFP-250, 5'-CCGGAGATCTCAGGGGAAATAATCTATTTGAA-3', S-mAFP-180, 5'-CCGGAGATCTACTCTGAAGTGGTCTTTGTC-3', and S-mAFP-90, 5'-CCGGAGATCTAATAGAGTCATATGTTTGCTCA-3', and a common downstream primer As-mAFP+25, 5'-CCGGAAGCTTGCAGGCAGTGCTGGAAGT-3', PCR reactions were carried out using the mouse tail DNA as a template. After digestion with *Bgl*II and *Hind*III, amplified DNA fragments were subcloned into the *Bgl*II/ *Hind*III sites of the pGL3-Basic to obtain pmAFP-250/Luc, pmAFP-180/Luc, and pmAFP-90/Luc, respectively. A 2.6–kb *Bgl*II fragment of the pDsRed1C1-hZHX2cFL was subcloned into the *Bam*HI site of the pCMV-Tag2B to produce pFLAG-ZHX2. The nucleotide sequences of all inserts were confirmed using a DNA sequencer 3100 (Applied Biosystems).

3.7. DNA transfections

All plasmids used for the transfection were prepared using a GenoPure plasmid maxi kit, followed by CsCl density gradient ultracentrifugation. SL2 cells were plated at a density of 1 x 10^6 cells per a 60 mm-dish. After 24 hours, 2 micrograms of a luciferase reporter plasmid, 100 ng of pPac-betaGal and the indicated amount of pPac-derived expression plasmids were transfected into SL2 cells using a calcium phosphate method (25). The total amount of DNA was adjusted by the addition of the pPac plasmid. The cells were harvested at 48 hours after transfection and firefly and beta-galactosidase activities were determined as described previously (26). Luciferase activities were determined on a Berthold Lumat model LB 9501 (Wildbad, Germany). Firefly luciferase activities (relative light units) were normalized by beta-galactosidase activities.

For HLE cells, DNA transfections were carried out using the Lipofectamine PLUS reagent. 5 x 10^4 cells per well were inoculated in a 24 well plate on the day prior to transfection. Two hundred ng of a reporter plasmid, 1 ng the pRL-CMV, the indicated amount of pFLAG-ZHX2 expression plasmid were used. The total amount of plasmid DNA (302 ng) was adjusted by the addition of pCMV-Tag2B, if necessary. Firefly and sea pansy luciferase assays were carried out using the dual luciferase assay system. Procedures were performed according to the manufacture's protocol. Firefly luciferase activities (relative light units) were normalized by sea pansy luciferase activities. Statistical differences were determined by a two-tailed Student's t-test.

3.8. Library screening

The pDBD-ZHX2 (1-837) and pDBD-ZHX3 (1-956) which express entire coding sequence of human ZHX2 and ZHX3, respectively, fused to the DNA-binding domain (DBD) of yeast transcription factor GAL4, and the construction of rat granulosa cell and liver cDNA libraries were described previously (2, 4, 6, 27). AH109 yeast cells were transformed with the pDBD-ZHX2 (1-837) or pDBD-ZHX3 (1-956) plasmid. Each strain was used as a bait to screen cDNA libraries. A TE/LiAc-based high efficiency transformation method was used for library screening (28). For screening of ZHX2- or ZHX3-interacting proteins, 2.4 x 10^6 and 3 x10^6 independent clones of the liver and granulosa cell cDNA libraries were plated on histidine-, tryptophan-, leucine-, and adenine-free synthetic dextrose plates supplemented with 1 mM 3-aminotriazole and X-alpha-gal, respectively.

As the ZHX2-interacting proteins, 21 and 44 positive clones were obtained from the primary transformants, respectively. The yeast strain SFY526 that contains a quantifiable *lacZ* reporter, and the pDBD-ZHX2 (1-873) plasmid, was transformed with plasmids isolated from positive clones in primary screening or the parent vector, pACT2. In the second screening, 7 and 19 clones from liver and granulosa cell cDNA libraries, respectively, exhibited reproducible high beta-galactosidase activity. Quantitative beta-galactosidase assays, using *o*-nitrophenyl-beta-D-galactoside, were carried out on permeabilized cells, as described previously (1, 5, 29). The yeast strain SFY526 that contains the pGBKT7 plasmid was transformed with plasmids isolated from positive clones in the second screening. In the third screening, 6 and 16 clones from liver and granulosa cell cDNA libraries, respectively, exhibited no beta-galactosidase activity.

As ZHX3-interacting proteins, 22 and 33 positive clones were obtained from the primary transformants of liver and granulosa cell cDNA libraries, respectively. The yeast strain SFY526 that contains a quantifiable *lacZ* reporter, and the pDBD-ZHX3 (1-956) plasmid, was transformed with plasmids isolated from positive clones in primary screening or the parent vector, pACT2. In the second screening, 8 and 20 clones from liver and granulosa cell cDNA libraries, respectively, exhibited reproducible high beta-galactosidase activity. In the third screening as well as screening of ZHX2-interacting proteins, 7 and 20 clones from liver and granulosa cell cDNA libraries, respectively, exhibited no beta-galactosidase activity

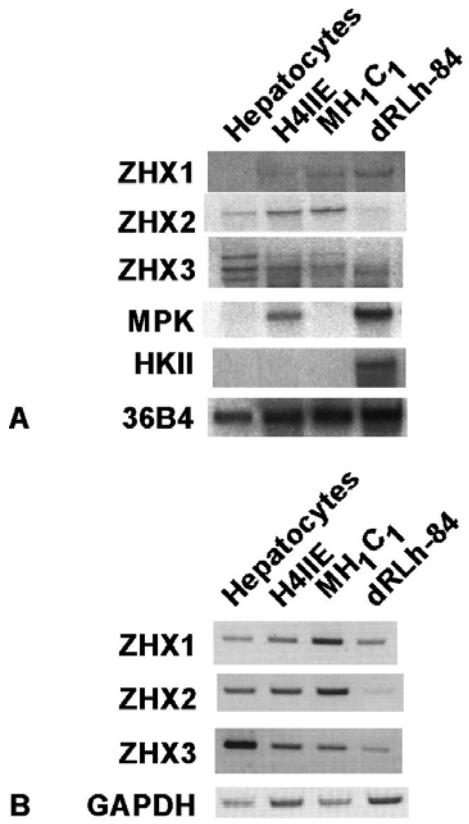

Figure 1. Expression of mRNAs of members of the ZHX family and various genes in isolated rat hepatocytes and rat hepatoma cell lines. (A) Five micrograms of polyA$^+$-RNA from isolated rat hepatocytes and various rat hepatoma cells shown at the *top* were subjected to a 0.8 % denaturating agarose gel electrophoresis and then transferred to a nylon membrane. The membranes were hybridized with ^{32}P-labeled probes shown on the *left*, respectively. (B) RT-PCRs were performed using total RNAs prepared from isolated rat hepatocytes and various rat hepatoma cells shown at the *top*. One microgram of total RNAs were reverse-transcribed, and a portion subjected to the PCR reaction for the specific amplification of genes shown on the *left*, respectively. The reaction mixture was subjected to a 2 % agarose gel electrophoresis, then visualized with ethidium bromide-staining. ZHX1, zinc-fingers and homeoboxes 1; ZHX2, zinc-fingers and homeoboxes 2; ZHX3, zinc-fingers and homeoboxes 3; MPK, M_2-type pyruvate kinase; HKII, type II hexokinase; 36B4, ribosomal protein 36B4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

4. RESULTS

4.1. Analysis of gene expression of members of the ZHX family in isolated rat hepatocytes and various hepatoma cell lines

To analyze the possible roles of members of the ZHX family in normal liver and hepatoma cells, the levels of these mRNAs in isolated hepatocytes and three rat hepatoma cell lines were determined by Northern blot (Figure 1A) and PCR (Figure 1B) analyses. While both H4IIE cells and MH_1C_1 cells are well-differentiated hepatoma cell lines expressing some liver-specific genes, dRLh-84 cells are a poorly-differentiated malignant hepatoma cell line that does not express any liver-specific genes (30).

Upon Northern blot analysis as shown in Figure 1A, both ZHX1 and ZHX2 mRNAs were expressed as a single band although multiple forms of ZHX3 mRNA from the use of several polyadenylation signals were observed as described previously (2, 4, 6). ZHX2 and ZHX3 expression levels were higher than that of ZHX1 in normal hepatocytes where both cancer markers PKM and HKII were repressed. In contrast to a high level of ZHX1 expression, the levels of both ZHX2 and ZHX3 mRNAs were quite low in malignant dRLh-84 cells where both PKM and HKII mRNAs were highly expressed, suggesting that ZHX2 and ZHX3 might be involved in the repression of PKM and HKII genes.

4.2. Members of the ZHX family regulate the expression of hepatocellular carcinoma biomarkers

In order to investigate the involvement of members of the ZHX family proteins in the aberrant expression of *PKM*, *HKII*, and *cdc25C* genes in hepatocellular carcinoma, we performed promoter assays using insect SL2 cells because this cell line is devoid of endogenous NF-Y, Sp1, and the members of the ZHX family (22, 24, 31). The luciferase reporter plasmids, which contain the nucleotide sequence between –287 and +46 of the rat *PKM* gene promoter (pMPK287/Luc), –157 and +147 of the rat *HKII* gene promoter (prHKII-157/Luc), and –172 and +10 of the human *cdc25C* gene promoter (pCDC25/Luc), were previously reported (12, 13, 32). Because the promoter activity of the rat *PKM* gene was synergistically increased by co-transfection of both Sp1- and NF-Y-expression vectors in SL2 cells, effects of ZHX1, ZHX2, and ZHX3 expression on the Sp1/NF-Y-dependent expression of luciferase gene mediated by the PKM promoter was examined (Figure 2A) (12). ZHX1 and ZHX3 reduced the Sp1/NF-Y-dependent activation of PKM promoter, but ZHX2 did not show any reduction. As previously reported, NF-Y activated the luciferase expression mediated by the rat *HKII* promoter (Figure 2B) (13). The transcriptional activation of luciferase gene by NF-Y was decreased by the expression of ZHX1, ZHX2, or ZHX3 although ZHX3 was most effective. The expression of the human *cdc25C* gene, a cell cycle-regulating gene, is also NF-Y-dependent (3). As shown in Figure 2C, promoter activity of the *cdc25C* gene stimulated by NF-Y was repressed by ZHX2 or ZHX3 but not ZHX1. These results indicate that members of the ZHX family negatively regulate the transcription of genes activated by NF-Y although each member might be involved in the different negative regulation of genes.

4.3. ZHX2 represses transcription from the mouse alpha-fetoprotein (AFP) gene promoter

It has been reported that lower expression of the mouse *ZHX2* gene causes high level of expression of the mouse *AFP* gene (33). Therefore, we examined effects of ZHX2 on the mouse *AFP* gene promoter. A

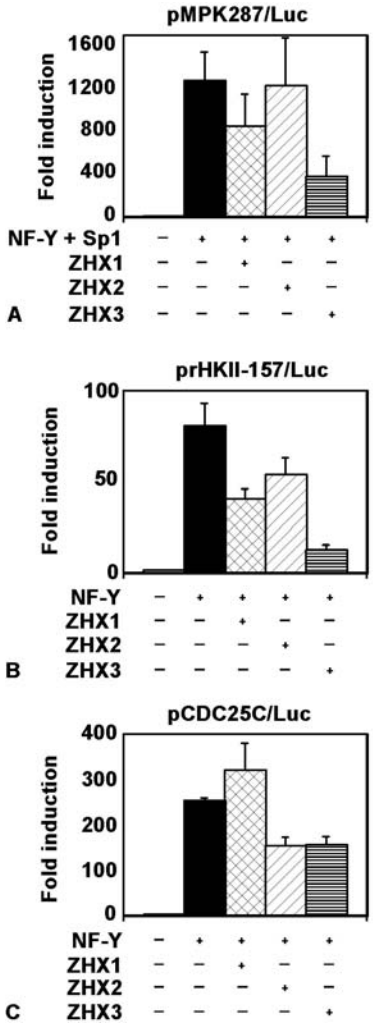

Figure 2. Transcriptional repression of members of the ZHX family on promoter activity of various genes stimulated by NF-Y with or without Sp1 in *Drosophila* SL2 cells. (A) Two micrograms of pMPK287/Luc reporter plasmid was co-transfected with 100 ng of pPac-betaGal, 100 ng of pPac or each 25 ng of pPacNF-YA, pPacNF-YB, pPacNF-YC, and pPac-Sp1 with 200 ng of pPac, pPac-ZHX1, pPac-ZHX2, or pPac-ZHX3 into SL2 cells. Total DNA amount (2.4 micrograms) was adjusted by the addition of the pPac plasmid. (B) Two micrograms of prHKII-157/Luc reporter plasmid or (C) Two micrograms of pCDC25/Luc was co-transfected with 100 ng of pPac-betaGal, 75 ng of pPac or each 25 ng of pPacNF-YA, pPacNF-YB, and pPacNF-YC with 200 ng of pPac, pPac-ZHX1, pPac-ZHX2, or pPac-ZHX3 into SL2 cells. Total DNA amount (2.375 micrograms) was adjusted by the addition of the pPac plasmid. At 48 hours after transfection, the cells were subjected to luciferase and beta-galactosidase assays. Each column and error bar represents the mean and standard error of at least four separate transfection experiments. The data are presented as fold stimulation where the value of luciferase activity normalized to beta-galactosidase activity for the reporter alone is set at 1.0.

cytomegalovirus enhancer/ promoter-directed ZHX2 expression plasmid, pFLAG-ZHX2, was co-transfected with two reporter plasmids into HLE cells. Nucleotide sequences between -250 and +25 of the mouse *AFP* gene and between -83 and +50 of the mouse *Zhx1* gene were inserted into a luciferase reporter plasmid to give plasmids pmAFP-250/Luc and pZHX1-83/Luc, respectively. When the pCMV-Tag2B plasmid, an empty vector for the ZHX2 expression plasmid, was transfected with the reporter plasmid, the relative luciferase activity was set to 100 %. As shown in Figure 3A, when the pmAFP-250/Luc was co-transfected with pFLAG-ZHX2, the luciferase activity was decreased in a concentration-dependent manner. Maximal inhibition was obtained with 100 ng of the pFLAG-ZHX2. In contrast, when the pZHX1-83/Luc plasmid was co-transfected with the pFLAG-ZHX2, the luciferase activity remained unchanged. It indicates that ZHX2 specifically represses the transcription of the mouse *AFP* gene promoter.

To investigate the transcriptional regulatory region of the mouse *AFP* gene by ZHX2, we constructed a series of 5'-deletion mutants of the mouse *AFP* gene promoter fused to the luciferase reporter plasmid (Figure 3B). When the deletion up to -181 (pmAFP-180/Luc) was co-transfected with FLAG-ZHX2 expression plasmid, luciferase activity was decreased to same level of that of the pmAFP-250/Luc. In contrast, the luciferase activities of a construct deleted up to -91 (pmAFP-90/Luc) and the pGL3-Basic, a promoter-less luciferase reporter vector, remained unchanged with a co-transfection of pFLAG-ZHX2. These results indicate that the nucleotide sequence between -180 and -91 of the mouse *AFP* gene responds to the repression by ZHX2.

4.4. Screening of ZHX2- or ZHX3-interacting proteins

To analyze the molecular mechanism of transcriptional repression by ZHX2 and ZHX3, we examined the issue of whether these proteins interact with either a known or a novel transcription factor. An entire coding sequence of the human ZHX2 or ZHX3 was fused to the GAL4 DBD and these chimeric proteins were employed as the bait to screen rat liver and granulosa cell cDNA libraries using the yeast two-hybrid system. Approximately 2.4 x 10^6 and 3 x 10^6 independent clones of each library were screened, and some clones showed reproducible His^+, Ade^+, and alpha-gal positive properties, respectively. We isolated plasmids that encode the GAL4 AD fusion protein from these clones. After determination of their nucleotide sequences, they were compared with the GenBank database using the BLAST search program. As shown in Tables 1 and 2, transcription factors containing ZHX1, protein kinase, guanine nucleotide exchange factors (GEFs), cytoskeletal proteins, and others in addition to unknown proteins, were cloned.

5. DISCUSSION

Transcription of both the *PKM* and *HKII* genes is inactive in the normal rat liver and active in malignant hepatoma cells. NF-Y is a common transcriptional

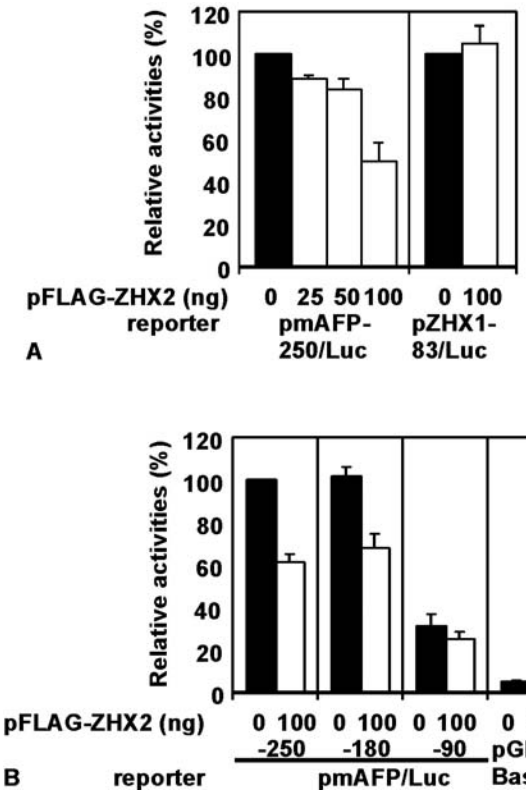

Figure 3. ZHX2 represses promoter activity of the mouse *AFP* gene. (A) HLE cells were co-transfected with 200 ng of the pmAFP-250/Luc or pZHX1-83/Luc, 1 ng of the pRL-CMV, 100 ng of the pCMV-Tag2B or the indicated amount of the pFLAG-ZHX2. The total amount of plasmid DNA (301 ng) was adjusted by the addition of the pCMV-Tag2B, if necessary. At 48 hours after transfection, the cells were subjected to luciferase assays. A value of 100 % was assigned to the promoter activity of each reporter plasmid in the presence of 100 ng of the pCMV-Tag2B. Each column and error bar represents the mean and standard error of at least three independent experiments. (B) HLE cells were co-transfected with 200 ng of the pmAFP-250/Luc, pmAFP-180/Luc, pmAFP-90/Luc, or pGL3-Basic, 1 ng of the pRL-CMV, 100 ng of the pCMV-Tag2B or pFLAG-ZHX2. At 48 hours after transfection, the cells were subjected to luciferase assays. A value of 100 % was assigned to the promoter activity of the pmAFP-250/Luc reporter plasmid in the presence of the pCMV-Tag2B. Each column and error bar represents the mean and standard error of at least three independent experiments.

activator for these gene promoters in cancer cells. To understand the silencing mechanism of both the *PKM* and *HKII* genes in normal liver, we have studied on members of the ZHX transcriptional repressor family which are NF-YA-interacting proteins. We reported herein on analysis of gene expression of members of the ZHX family in the isolated rat hepatocytes and hepatoma cell lines, effects of transcriptional repression of their family proteins on various gene promoters, and molecular cloning of ZHX2- and ZHX3-interacting proteins.

While neither M_2-PK nor HKII mRNAs were expressed in isolated rat hepatocytes and MH_1C_1 cells, M_2-PK mRNA was expressed in H4IIE cells and both M_2-PK and HKII mRNAs were expressed in dRL-h84 cells (Figure 1). Of these, the dRLh-84 cells are most malignant hepatoma cells. The levels of ZHX2 and ZHX3 mRNAs but not ZHX1 mRNA were decreased in dRLh-84 cells. Malignancy of hepatoma cells may result in low level of expression of the *ZHX2* and *ZHX3* genes. Indeed, a decrease in expression of the *ZHX2* gene was observed in some malignant cancer cells (34, 35).

Members of the ZHX family repressed promoter activities of various genes in a promoter-dependent manner (Figure 2). ZHX1 repressed promoter activities of both the *PKM* and *HKII* genes and ZHX2 repressed those of both *HKII* and *cdc25C* genes. In contrast, ZHX3 repressed all gene promoters examined. An increase of promoter activity of the *PKM* gene by co-transfection of the NF-Y expression vectors alone in SL2 cells was only two-fold (12). However, in the presence of Sp1- or Sp3-expression vector, the activity increased to over 1,200-fold (Figure 2A and (12)). Therefore, NF-Y and members of the Sp family protein synergistically enhance promoter activity of the *PKM* gene. In contrast, promoter activities of the *HKII* and *cdc25C* gene strongly increased by co-transfection of NF-Y expression vectors alone in SL2 cells. In the latter two cases, ZHX2 showed repressor activities (Figure 2), indicating that a degree of NF-Y-dependency on promoter activity may be a reason of difference of ZHX2 effect among promoters.

It has been reported that lower expression of the mouse *ZHX2* gene causes high level of expression of the mouse *AFP* gene (33). In addition, expression of the oncofetal glypican 3 gene is repressed by ZHX2 (36). These gene as well as the *PKM* and *HKII* genes is repressed in the liver after birth and reactivated in hepatocellular carcinogenesis (37). In HLE hepatoma cells, ZHX2 specifically repressed promoter activity of the mouse *AFP* gene (Figure 3). The nucleotide sequence between -180 and -91 of the mouse *AFP* gene is responsible for a repression activity by ZHX2. Recently, it has been reported that ZHX2 represses promoter activity of the human *AFP* gene via hepatocyte nuclear factor 1 (HNF1)-binding sites (38). The promoter region of the mouse *AFP* gene identified in our results corresponds to a region containing HNF1-binding sites of the human one. It suggests that (a) transcription factors other than NF-Y are also involved in the transcriptional repression by ZHX2.

Lastly, to address the issues whether ZHX2 and ZHX3 interact with protein (s) other than NF-Y, we searched ZHX2- and ZHX3-interacting protein (s) using a yeast two-hybrid system (Tables 1 and 2). As the ZHX2-interacting proteins, ZHX1, Atxn1, XM_001077702 were cloned. These proteins also interacted with ZHX1 (4). In addition, Hcfc and GABPB2 as transcription factors, Zfp131 and Zfp198 as zinc-finger proteins, Grasp1 and Plekhg2 as GEFs, HR21spA and LCP1 nuclear proteins, fibronectin, filamin beta, Lamin B1as cytoskeletal proteins, neogenin and Ewsr1 as cell growth-related proteins, and so on were cloned (Table 1). As the ZHX3-interacting

Table 1. The ZHX2-interacting proteins

Protein	Number of clones	Accession number
From liver cDNA library		
ZHX1	1	NM_133620
Hcfc	1	XM_343843
Rock1	1	NM_031098
Atxn1	1	NM_012726
Fibronectin	1	NM_019143
Filamin beta	1	NM_001107288
From granulosa cell cDNA library		
GABPB2	1	NM_001039036
LCP1	1	NM_173324
HR21spA	2	NM_00102570
Zfp131	2	XM_001076567
Zfp198	1	XM_001060431
Neogenin	1	U68726
PRKA	1	NM_194462
Grasp1	1	NM_053807
Plekhg2	1	XM_214862
Ewsr1	1	BC098822
Lamin B1	1	NM_053905
Others	1	XM_230778
Others	1	XM_001062691
Others	1	XM_001077702

Table 2. The ZHX3-interacting proteins

Protein	Number of clones	Accession number
From liver cDNA library		
BRD3	2	NM_001108575
BRD4	2	XM_343175
Zyxin	1	XM_216124
Fibronectin	2	NM_019143
From granulosa cell cDNA library		
Protein	Number of clones	Accession number
BRD2	3	NM_212495
BRD3	4	NM_001108575
BRD4	1	XM_216124
Zyxin	3	XM_216124
Beta-actin	3	NM_031144
Filamin alpha	1	XM_238167
Lamin B1	1	NM_053905
Neogenin	1	U68726
Hipk1	1	EF460311
Mrip	1	NM_053814
GlcNac	1	NM_017107

proteins, zyxin was cloned. This protein was also cloned as a ZHX1-interacting protein (4). In addition, BRD2, BRD3, BRD4 as bromodomain-containing proteins, fibronectin, filamin alpha, Lamin B1, Mrip, and beta-actin as cytoskeletal proteins, Hipk1 as protein kinase, neogenin as a cell growth-related protein, and so on were cloned (Table 2). The issue of the nature of the biological significance of these interactions remains to be determined.

In summary, we have shown that the levels of both ZHX2 and ZHX3 mRNAs were decreased in malignant hepatoma cells, members of the ZHX family differentially regulate promoter activity of the genes which express in a hepatoma cell-specific manner, ZHX2 represses promoter activity of the AFP gene via a region containing HNF1-binding sites, and that ZHX2- and ZHX3-interacting proteins were cloned. Further studies will be required to completely understand the biological role of members of the ZHX family, particularly detailed analysis of their interactions with ZHX2- and ZHX3-interacting proteins.

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7. REFERENCES

1. K. Yamada, R. L. Printz, H. Osawa, and D. K. Granner: Human ZHX1: cloning, chromosomal location, and interaction with transcription factor NF-Y. *Biochem. Biophys. Res. Commun.* 261, 614-621 (1999)

2. H. Kawata, K. Yamada, Z. Shou, T. Mizutani, T. Yazawa, M. Yoshino, T. Sekiguchi, T. Kajitani, and K. Miyamoto: Zinc-fingers and homeoboxes (ZHX) 2, a novel member of the ZHX family, function as a transcriptional repressor. *Biochem J.* 373, 747-757 (2003)

3. H. Kawata, K. Yamada, Z. Shou, T. Mizutani, and K. Miyamoto: The mouse zinc-fingers and homeoboxes (ZHX) family: ZHX2 forms a heterodimer with ZHX3. *Gene* 323, 133-140 (2003)

4. K. Yamada, H. Kawata, Z. Shou, S. Hirano, T. Mizutani, T. Yazawa, T. Sekiguchi, M. Yoshino, T. Kajitani, and K. Miyamoto: Analysis of zinc-fingers and homeoboxes (ZHX1) 1-interacting proteins: molecular cloning and characterization of a member of the ZHX family, ZHX3. *Biochem J.* 373, 167-178 (2003)

5. K. Yamada, H. Osawa, and D. K. Granner: Identification of proteins that interact with NF-YA. *FEBS Lett.* 460, 41-45 (1999)

6. S. Hirano, K. Yamada, H. Kawata, Z. Shou, T. Mizutani, T. Yazawa, T. Kajitani, T. Sekiguchi, M. Yoshino, Y. Shigematsu, M. Mayumi, and K. Miyamoto: Rat zinc-fingers and homeoboxes 1 (ZHX1), a nuclear factor-YA-interacting nuclear protein, forms a homodimer. *Gene* 290, 107-114 (2002)

7. K. Yamada, H. Kawata, K. Matsuura, Z. Shou, S. Hirano, T. Mizutani, T. Yazawa, M. Yoshino, T. Sekiguchi, T. Kajitani, and K. Miyamoto: Functional analysis and the molecular dissection of zinc-fingers and homeoboxes 1 (ZHX1). *Biochem. Biophys. Res. Comm.* 279, 368-374 (2002)

8. G. Liu, L. C. Clement, Y. S. Kanwar, C. Avila-Casado, and S. S. Chugh: ZHX proteins regulate podocyte gene expression during the development of nephrotic syndrome. *J. Biol. Chem.* 281, 39681-39692 (2006)

9. K. Yamada, and T. Noguchi: Nutrient and hormonal regulation of pyruvate kinase gene expression. *Biochem. J.* 337, 1-11 (1999)

10. K. Yamada, and T. Noguchi: Regulation of pyruvate kinase M gene expression. *Biochem. Biophys. Res. Commun.* 256, 257-262 (1999)

11. R. L. Printz, H. Osawa, H. Ardehali, S. Koch, and D. K. Granner: Hexokinase II gene: strcture, regulation and promoter organization. *Biochem. Soc. Trans.* 25, 107-112 (1997)

12. K. Yamada, T. Tanaka, K. Miyamoto, and T. Noguchi: Sp family members and nuclear factor-Y cooperatively stimulate transcription from the rat pyruvate kinase M gene distal promoter region via their direct interactions. *J. Biol. Chem.* 275, 18129-18137 (2000)

13. H. Osawa, R. B. Robey, R. L. Printz, and D. K. Granner: Identification and characterization of basal and cyclic AMP response elements in the promoter of the rat hexokinase II gene. *J. Biol. Chem.* 271, 17296-17303 (1996)

14. J. L. Harousseau, J. J. Shaughnessy, and P. Richardson: Multiple myeloma. *Hematology 2004*, 237-256 (2004)

15. J. Sato, T. Yabe, and E. Chikata: Carcinogenesis in tissue VI: tissue culture of liver cells from DAB-feeding rats. *Jpn. J. Exp. Med.* 35, 491-511 (1965)

16. I. Doi, M. Namba, and J. Sato: Establishment and some biological characteristics of human hepatoma cell lines. *Gann* 66, 385-392 (1975)

17. P. O. Seglen: Preparation of isolated rat liver cells. *Methods Cell Biol.* 13, 29-83 (1976)

18. M. Takenaka, T. Noguchi, H. Inoue, K. Yamada, T. Matsuda, and T. Tanaka: Rat pyruvate kinase M gene: its complete structure and characterization of the 5'-flanking region. *J. Biol. Chem.* 264, 2363-2367 (1989)

19. Y. Shinohara, J. Ichihara, and H. Terada: Remarkably enhanced expression of the type II hexokinase in rat hepatma cell line AH130. *FEBS Lett.* 291, 55-57 (1991)

20. K. Yamada, H. Kawata, Z. Shou, T. Mizutani, T. Noguchi, and K. Miyamoto: Insulin induces the expression of the *SHARP-2/Stra13/DEC1* gene via a phosphoinositide 3-kinase pathway. *J. Biol. Chem.* 278, 30719-30724 (2003)

21. Z. Shou, K. Yamada, T. Inazu, H. Kawata, S. Hirano, T. Mizutani, T. Yazawa, T. Sekiguchi, M. Yoshino, T. Kajitani, K. Okada, and K. Miyamoto: Genomic structure and analysis of transcriptional regulation of the mouse zinc-fingers and homeoboxes 1 (*ZHX1*) gene. *Gene* 302, 83-94 (2003)

22. A. J. Courey, D. A. Holtzman, S. P. Jackson, and R. Tjian: Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. *Cell* 59, 827-836 (1988)

23. G. Hagen, S. Muller, M. Beato, and G. Suske: Sp1-mediated transcriptional activation is repressed by Sp3. *EMBO J.* 13, 3843-3851 (1994)

24. K. A. Dooley, M. K. Bennett, and T. F. Osborne: A critical role for cAMP response element-binding protein (CREB) as a co-activator in sterol-regulated transcription of 3-hydroxy-3-methylglutaryl coenzyme A synthase promoter. *J. Biol. Chem.* 274, 5285-5291 (1999)

25. P. P. Di Nocera, and I. B. Dawid: Transient expression of genes introduced into cultured cells of *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 80, 7095-7098 (1983)

26. E. Kikkawa, M. Hinata, V. W. Keng, Z. Myint, A. Sato, K. Yamada, T. Tanaka, and T. Noguchi: Sp family members stimulate the transcription of Hex gene via interactions with GC boxes. *J. Biochem. (Tokyo)* 130, 885-891 (2001)

27. K. Yamada, T. Mizutani, Z. Shou, T. Yazawa, T. Sekiguchi, M. Yoshino, T. Inazu, and K. Miyamoto: Cloning and functional expression of an E box-binding protein from rat granulosa cells**.** *Biol. Reprod.* 64, 1315-1319 (2001)

28. K. Yamada, J.-C. Wang, H. Osawa, D. K. Scott, and D. K. Granner: Efficient large-scale transformation of yeast. *BioTechniques* 24, 596-600 (1998)

29. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, 13. Saccharomyces cerevisiae., in Current protocols in molecular biology, in Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., (Eds.), John Wiley and Sons, New York, 1994, pp. 13.16.11-13.16.16.

30. K. Imamura, T. Noguchi, and T. Tanaka, Regulation of isozyme patterns of pyruvate kinase in normal and neoplastic tissues., in Markers of Human Neuroectodermal Tumors, in Staal, G. E. J., and van Veelen, C. W. M., (Eds.), CRC Press, Boca Raton, 1986, pp. 191-222.

31. D. Bayarsaihan, B. Enkhmandakh, A. Makeyev, J. M. Greally, J. F. Leckman, and F. H. Ruddle: *Homez*, a homeobox leucine zipper gene specific to the vertebrate lineage. *Proc. Nat. Acad. Sci. U.S.A.* 100, 10358-10363 (2003)

32. J. Zwicker, C. Gross, F. C. Lucibello, M. Truss, F. Ehlert, K. Engeland, and R. Muller: Cell cycle regulation of cdc25C transcription is mediated by the periodic repression of the glutamine-rich activators NF-Y and Sp1. *Nucleic Acids Res.* 23, 3822-3830 (1995)

33. S. Perincheri, C. R. W. Dingle, M. L. Peterson, and B. T. Spear: Hereditary persistence of α-fetoprotein and *H19* expression in liver of BALB/cJ mice is due to a retrovirus insertion in the *Zhx2* gene. *Proc. Nat. Acad. Sci. U.S.A.* 102, 396-401 (2005)

34. A. Armellini, M. E. Sarasquete, R. Garcia-Sanz, M. C. Chillon, A. Balanzategui, M. Alcoceba, M. Fuertes, R. Lopez, J. M. Hernandez, J. Fernandez-Calvo, M. Sierra, M. Megido, A. Orfao, N. C. Gutierrez, M. Gonzalez, and J. F. San Miguel: Low expression of ZHX2, but not RCBTB2 or RAN, is associated with poor outcome in multiple myeloma. *Br. J. Haematol.* 141, 212-215 (2008)

35. Z. Lv, M. Zhang, J. Bi, F. Xu, S. Hu, and J. Wen: Promoter hypermethylation of a novel gene, ZHX2, in hepatocellular carcinoma. *Am. J. Clin. Pathol.* 125, 740-746 (2006)

36. L. A. Morford, C. Davis, L. Jin, A. Dobierzewska, M. L. Peterson, and B. T. Spear: The oncofetal gene glypican 3 is regulated in the postnatal liver by zinc fingers and homeoboxes 2 and in the regenerating liver by alpha-fetoprotein regulator 2. *Hepatology* 46, 1541-1547 (2007)

37. B. T. Spear, L. Jin, S. Ramasamy, and A. Dobierzewska: Transcriptional control in the mammalian liver: liver development, perinatal repression, and zonal gene regulation. *Cell Mol. Life Sci.*, **(**2007)

38. H. Shen, F. Luan, H. Liu, L. Gao, X. Liang, L. Zhang, W. Sun, and C. Ma: ZHX2 is a repressor of alpha-fetoprotein expression in human hepatoma cell lines. *J. Cell. Mol. Med.*, (2008)

Abbreviations: NF-Y, nuclear factor-Y; PKM, M_2-type pyruvate kinase; HKII, type II hexokinase; DMEM, Dulbecco's modified Eagle's medium; SL2, Schneider line 2; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DBD, DNA-binding domain; AFP, alpha-fetoprotein; GEF, guanine nucleotide exchange factor; HNF1, hepatocyte nuclear factor 1.

Key Words: The ZHX family, Transcriptional Repressor, Cancer; cDNA Cloning, Two-Hybrid System, Pyruvate Kinase, Hexokinase, Liver

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