Zinc-fingers and homeoboxes (ZHX) 2, a novel member of the ZHX family, functions as a transcriptional repressor

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Zinc-fingers and homeoboxes (ZHX) 1 is a transcription factor that interacts with the activation domain of the A subunit of nuclear factor-Y (NF-YA). Using a yeast two-hybrid system, a novel ubiquitous transcription factor ZHX2 as a ZHX1-interacting protein was cloned. ZHX2 consists of 837 amino acid residues and contains two zinc-finger motifs and five homeodomains (HDs) as well as ZHX1. The mRNA is expressed among various tissues. ZHX2 not only forms a heterodimer with ZHX1, but also forms a homodimer. Moreover, ZHX2 interacts with the activation domain of NF-YA. Further analysis revealed that ZHX2 is a transcriptional repressor that is localized in the nuclei. Since ZHX2 shares a number of properties in common with ZHX1, we conclude that all these come under the ZHX family. The minimal functional domains of ZHX2 were then characterized. The dimerization domain with both ZHX1 and ZHX2 is the region containing HD1, the domain that interacts with NF-YA is the HD1 to HD2 region, the repressor domain is the HD1 to a proline-rich region. Lastly, using an immunoprecipitation assay, we showed that ZHX2 intrinsically interacts with NF-YA in HEK-293 cells and that ZHX2 represses the promoter activity of the *cdc25C* gene stimulated by NF-Y in *Drosophila* Schneider line 2 cells. Thus the ZHX family of proteins may participate in the expression of a number of NF-Y-regulated genes via a more organized transcription network.

Key words: dimerization, nuclear factor-Y, nuclear localization signal, transcriptional repressor, zinc-fingers and homeoboxes 1 (ZHX1).

INTRODUCTION

Transcription factors bind to the cognate DNA sequence in the promoter region of the gene to regulate transcription positively or negatively. The existence of cell type-specific transcription factors and ubiquitous transcription factors has been reported [1]. Unique combinations of these factors on the promoter serve to direct gene-specific transcription. In addition to these DNA-binding proteins, DNA-non-binding proteins also regulate the transcription of target genes. These factors, which are referred to as co-activators or co-repressors, act by bridging or interfering with interactions among DNA-binding proteins and the basic transcription machinery [2,3].

An ubiquitous transcription factor, nuclear factor-Y (NF-Y), binds to an inverted CCAAT box (also referred to as the Y box, 5′-ATTGG-3′), stimulating the transcription of a number of genes [4]. This factor consists of three subunits, NF-YA, NF-YB and NF-YC. These subunits not only interact with one another, but also interact with other transcription factors. For example, NF-YA interacts with hepatocyte nuclear factor 4, NF-YB interacts with p300 (a transcriptional activator protein required to drive p53 expression) or the TATA-box-binding protein and the NF-YC interacts with the TATA-box-binding protein [4] respectively.We reported previously that NF-Y stimulates the transcription of the rat pyruvate kinase M gene, and that physical interactions of NF-YA with specificity protein 1 (Sp1) and Sp3 are important for the synergistic activation of transcription [5]. It has also been reported previously [6] that NF-YA interacts with the p300/cAMP-response-element-binding protein-associated factor (P/CAF), a transcriptional co-activator with intrinsic histone acetyltransferase activity.

Human zinc-fingers and homeoboxes (ZHX) 1 was cloned as a protein that interacts with the activation domain (AD) of NF-YA [7,8]. Another group reported independently that mouse ZHX1, acting as antigen, is recognized by the B92 monoclonal antibody produced by immunization with a cell lysate of 14F1.1 endothelial-adipose stromal cells [9]. We reported recently on the cloning of rat ZHX1 as a ZHX1-interacting protein, which suggests that ZHX1 is capable of dimerizing [10]. All the ZHX1 molecules are comprised of 873 amino acid residues and contain two Cys-Xaa$_2$-Cys-Xaa$_{12}$-His-Xaa$_4$-His-type zinc-finger (Znf) motifs and five homeodomains (HDs) from the N-terminus [7,9,10]. They belong to the Znf class of the homeobox protein superfamily [11]. ZHX1 mRNA is expressed in various tissues at different levels [7,9,10]. An interaction of ZHX1 with NF-YA requires an amino acid sequence corresponding to the HD1 to HD2 region of ZHX1. In contrast, a glutamine-rich region in the AD of NF-YA interacts with ZHX1 [8]. An entire coding sequence of ZHX1 fused to the green fluorescent protein (GFP) is localized in the nuclei in HEK-293 cells [10]. We also showed recently that ZHX1 is a transcriptional repressor [12].

NF-Y is thought to be essential for oestradiol-stimulated cell proliferation in MCF-7 cells through the activation of various cell-cycle-related genes, including *cdc25C* [13,14]. It has also been

Abbreviations used: AD, activation domain; DBD, DNA-binding domain; GFP, green fluorescent protein; GST, glutathione S-transferase; HD, homeodomain; NF-Y, nuclear factor-Y; NLS, nuclear localization signal; P/CAF, p300/cAMP-response-element-binding protein-associated factor; RT, reverse transcriptase; SL2, Schneider line 2; Sp, specificity protein; SV40, simian virus 40; ZHX, zinc-fingers and homeoboxes; Znf, zinc finger.

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Nucleotide sequences of pAD-L26 and the entire coding of human ZHX2 cDNA have been submitted to the DNA Databank of Japan under the accession numbers AB081946 and AB083653 respectively.

reported that the stable expression of the dominant-negative form of NF-YA results in the retardation of growth of mouse fibroblast cells [15]. This form is not capable of binding to the cognate nucleotide sequence, but is able to associate with ZHX1. In addition, the level of ZHX1 mRNA temporarily increases within 6 h, in the case of mouse T-cells treated with interleukin-2 [16]. These findings suggest that ZHX1 with NF-Y may regulate the gene expression involved in cell growth and differentiation.

To gain further insight into the biological role of ZHX1, the present study was conducted to address the issue of whether ZHX1 interacts with protein(s) other than NF-YA. In the present study, we show the molecular cloning of the novel transcriptional repressor ZHX2 as a ZHX1-interacting protein. ZHX2 consists of 837 amino acid residues and has two Cys_2-His_2-type Znf motifs and five HDs as well as ZHX1. We then characterized the properties of the ZHX2 transcriptional factor. These observations indicate that both ZHX1 and ZHX2 comprise a family of proteins, the ZHX family. In addition, we show the intrinsic interaction of ZHX2 with NF-YA in HEK-293 cells and the repressor activity of ZHX2 on the promoter activity of an NF-Y-regulated gene, *cdc25C*.

EXPERIMENTAL

Materials

The yeast two-hybrid system, X-α-gal, pEGFP-C1, human genomic DNA, human multiple-tissue Northern blot and ExpressHyb hybridization solution were purchased from Clontech (Palo Alto, CA, U.S.A.). HEK-293 cells, a human embryonic kidney cell line, were purchased from A.T.C.C. (Manassas, VA, U.S.A.). The TRIzol® reagent, Superscript II, pcDNA3.1 His C, LIPOFECTAMINE™ PLUS, Schneider's medium and Calcium Phosphate Transfection kit were purchased from Invitrogen (Groningen, The Netherlands). The ExTaq DNA polymerase and BcaBest DNA labelling kit were obtained from Takara Biomedicals (Kyoto, Japan). The pGEM-T Easy vector, pGL3-Basic, T7 TNT Quick-coupled transcription/translation system, pGL3 control, pRL-CMV and dual luciferase assay system were purchased from Promega (Madison, WI, U.S.A.). The pGEX-4T-2, pGEX-5X-1, glutathione–Sepharose 4B, [^{35}S]-methionine (37 TBq/mmol), Protein A–Sepharose 4 Fast Flow and ECL® Plus Western blotting reagent pack were purchased from Amersham Pharmacia Biotech (Cleveland, OH, U.S.A.). The Big Dye terminator FS cycle sequencing kit was purchased from Applied Biosystems (Tokyo, Japan). [α-^{32}P]dCTP (111 TBq/mmol) was purchased from NEN Life Science Products (Wilmington, DE, U.S.A.). TOPP3 cells were obtained from Stratagene (La Jolla, CA, U.S.A.). The Qiagen plasmid kit was purchased from Qiagen (Hilden, Germany). The SulfoLink kit was purchased from Pierce Biotechnology (Rockford, IL, U.S.A.). Anti-NF-YA polyclonal antibody (SC-10779) and anti-GST (where GST stands for glutathione S-transferase) polyclonal antibody (SC-459) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Immobilon-P, a PVDF membrane, was purchased from Millipore (Bedford, MA, U.S.A.).

Library screening

The pDBD-ZHX1 (1–873) [referred to previously as pGBKT7-ZHX1 (1–873)], which expresses the entire coding region of the human ZHX1 fused to GAL4 DNA-binding domain (DBD), and pACT2B1 vector have been described previously [8,10]. AH109 yeast cells were transformed with the pDBD-ZHX1 (1–873) plasmid. The yeast strain was employed as a bait to screen a rat liver cDNA library using a Tris/EDTA/lithium acetate-based high-efficiency transformation method [17,18]. Approx. 15 million independent clones of the cDNA library were plated on histidine-, tryptophan-, leucine- and adenine-free synthetic dextrose plates supplemented with 4 mM 3-aminotriazole and X-α-gal. Thirty-three positive clones from the primary transformants were obtained. The yeast strain SFY526 contains a quantifiable *lacZ* reporter gene. For the second screening, yeast, harbouring either the pGBKT7 or pDBD-ZHX1 (1–873), was transformed with plasmids isolated from the positive clones identified in the primary screening or the parent vector, pACT2. Their β-galactosidase activities were then determined. Quantitative β-galactosidase assays, using *o*-nitrophenyl-β-D-galactoside as a substrate, were performed on permeabilized cells, as described previously [7,8,10].

Plasmid construction

The pBSII-KIAA0854 was a gift from Dr Takahiro Nagase (Kazusa DNA Research Institute, Chiba, Japan). Total RNA from HEK-293 cells was prepared using the TRIzol® reagent according to the manufacturer's instructions. The pBSII-KIAA0854 plasmid and total RNA from HEK-293 were used as a template for PCRs and reverse transcriptase (RT)–PCR respectively. Using combinations of ZHX2 primers, PCR or RT–PCR was performed as described previously with minor modifications [7,8]. All amplified DNAs were subcloned into the pGEM-T Easy. The nucleotide sequences of all inserts were confirmed by sequencing.

The pGBKT7B1, pSG424, pSG424B1, 5 × GAL4-pGL3 control, pCMV-ZHX1 (272–432) and pEGFP-C1E1 plasmids have been described previously [19–23]. Oligonucleotides 5′-TATG-CCG-3′ and 5′-AATTCGGCA-3′ were annealed, phosphorylated and subcloned into *Nde*I/*Eco*RI sites of pSG424B1 to obtain pSG424B1E1A. Various PCR or RT–PCR products were digested with restriction enzymes and subcloned into the following vectors for each purpose. The nomenclature used for our constructs are as follows: the GAL4 DBD-fusion protein expression vector in yeast, pDBD-ZHX2; the GAL4 AD-fusion protein expression vector in yeast, pAD-ZHX2; the GST-fusion protein expression vector, pGST-ZHX2; the GAL4 DBD-fusion protein expression vector in mammal, pGAL4-ZHX2; the GFP-fusion protein expression vector, pGFP-ZHX2; and the *Drosophila* expression vector, pPac-ZHX2. The number described in the name of the plasmids except for clone L26 corresponds to the number of amino acid residues of human ZHX2. For example, the pGST-ZHX2 (263–497) encodes the amino acid sequence between residues 263 and 497 of ZHX2 fused to the C-terminal of GST. For detailed information concerning primers and constructs, contact the corresponding author of the present paper.

The pAD-ZHX1 (142–873) (referred to previously as pACT2 #111), pAD-ZHX1 (272–873), pAD-ZHX1 (565–873), pAD-ZHX1 (272–564), pAD-ZHX1 (272–432), pAD-ZHX1 (430–564), pAD-ZHX1 (345–463), pDBD, pYA1–112, pYA1–140, pYA141–269, pYA172–269, pYA205–269 and pYA1–269 were described previously [pAD-ZHX1 and pDBD constructs were referred to previously as pZHX1 and pGBT9 (N/R/N) constructs respectively] [7,8].

Genomic PCR was performed using human genomic DNA with a combination of oligonucleotides, 5′-CCGGAGATCTG-CTCTGGCTATGGGGCG-3′ and 5′-CCGGAAGCTTGGCGTT-GACCATTCAAAC-3′ as primers. An amplified DNA was subcloned into the pGL3-Basic to obtain pCDC25/Luc.

The pPac and pPac-Sp1 were generously provided by Dr Guntram Suske (Philipps-Universität Marburg, Germany)

[24,25]. The pPac-β-Gal, pPacNF-YA, pPacNF-YB and pPacNF-YC plasmids were gifts from Dr Timothy F. Osborne (University of California, Irvine, CA, U.S.A.) [26]. The pPac-Sp1 was digested with *Bam*HI and a large portion of the insert was removed. A fragment containing full-length human ZHX2 was subcloned into the resulting plasmid to produce pPac-ZHX2.

The nucleotide sequences of all inserts were confirmed by sequencing.

Polyadenylated-RNA-blot analysis

Human multiple-tissue Northern blot was hybridized with a 300 bp *Eco*RI fragment of the pBSII-KIAA0854, labelled with [α-^{32}P]dCTP using the BcaBest DNA labelling kit. For prehybridization and hybridization, the ExpressHyb hybridization solution was used. Conditions of prehybridization, hybridization and washing procedures were performed according to the manufacturer's instructions. The blot was exposed to a FUJIX imaging plate. Hybridization signals were detected with the FUJIX BAS-2000 image analysing system.

Yeast two-hybrid system

To analyse the dimerization domain of ZHX2, SFY526 yeast strains harbouring pDBD, pDBD-ZHX2 (263–322), pDBD-L26 or pDBD-ZHX2 (1–837) were employed. For determination of the heterodimerization domain with ZHX1, these reporter yeast strains were transformed with various truncated forms of ZHX1 fused to GAL4 AD or pACT2. To map the homodimerization domain, one among pACT2, pAD-ZHX2 (263–322), pAD-L26 and pAD-ZHX2 (1–837) was transformed into the reporter yeasts.

To examine the interaction domain of ZHX2 with NF-YA, SFY526 yeast strains harbouring the pDBD or pYA1–269 were transformed with four plasmids, the pACT2, pAD-L26, pAD-ZHX2 (263–497) and pAD-ZHX2 (1–837). For mapping the interacting domain of NF-YA with ZHX2, an SFY526 yeast strain harbouring pAD-ZHX2 (263–497) was transformed with various truncated forms of NF-YA fused to GAL4 DBD.

β-Galactosidase activities were determined as described previously [7,8,10].

GST pull-down assays

TOPP3 cells were transformed with the pGEX-5X-1, pGST-ZHX2 (263–497) or pGST-ZHX2 (1–837) fusion protein expression plasmids respectively. The preparation of the GST-fusion protein and *in vitro*-translated, ^{35}S-labelled ZHX1, have been described previously [7,10]. The pDBD-ZHX2 (1–837) was employed for the preparation of *in vitro*-translated, ^{35}S-labelled, ZHX2. Finally, the beads were resuspended in an equal volume of 2 $\times$ SDS sample buffer and each supernatant was subjected to SDS/PAGE [10 % (w/v) gel], along with a prestained molecular-mass marker. The gel was dried and exposed to a FUJIX imaging plate (Kanagawa, Japan). Interaction signals were detected using the FUJIX BAS-2000 image analysing system. The relative purity and amounts of each fusion protein were determined by gel-staining with Coomassie Brilliant Blue R-250.

Cell culture and DNA transfection

HEK-293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal bovine serum at 37 °C in a 5 % CO_2 incubator. In a 24-well plate, 5×10^4cells per well were inoculated on the day before transfection. DNA transfections were performed using the LIPOFECTAMINE™ PLUS reagent according to the method provided by the supplier.

For a determination of transcriptional activity of ZHX2, the 5 $\times$ GAL4-pGL3 control and pGL3 control were used as reporter plasmid. Reporter plasmid (100 ng), pRL-CMV (2 ng) and the indicated amount of GAL4 DBD–ZHX2-fusion protein expression plasmid were used. The total amount of plasmid (152 ng) was adjusted by the addition of the pSG424, whenever necessary. To analyse whether the heterodimerization of ZHX1 and ZHX2 is essential for the transcriptional activity of ZHX2, the 5 $\times$ GAL4-pGL3 control was used as the reporter plasmid: 100 ng of a reporter plasmid, 2 ng of the pRL-CMV, 50 ng of pGAL4-ZHX2 (1–837) fusion protein expression plasmid, and the indicated amount of pCMV-ZHX1 (272–432) plasmid were used. The total amount of plasmid (202 ng) was adjusted by the addition of pcDNA3.1 His C. The medium was changed 3 h after transfection. After 48 h, a luciferase assay was performed.

To observe the GFP-fusion protein, 300 ng of indicated GFP-fusion protein expression plasmids were transfected. The medium was changed 3 h after transfection. The cells were observed with a fluorescence microscope 72 h after transfection (Olympus IX-70) [10,20].

Schneider line 2 (SL2) cells, a *Drosophila* cell line, were a gift from Dr Tamio Noguchi (Nagoya University, Nagoya, Japan). SL2 cells were grown in Schneider's medium, supplemented with 10 % foetal bovine serum at 25 °C. Cells were plated at a density of 1×10^6 cells per 60 mm dish. After 24 h, 2 μg of luciferase reporter plasmid, 100 ng of pPac-β-Gal and the indicated amount of pPac-derived expression plasmids were transfected into SL2 cells using a calcium phosphate method [27]. The total amount of DNA (2.325 μg) was adjusted by the addition of the pPac plasmid. The cells were harvested at 48 h after transfection and subjected to luciferase and β-galactosidase assays.

All plasmids used for the transfection were prepared using a Qiagen plasmid kit, followed by CsCl density-gradient ultracentrifugation.

Luciferase and β-galactosidase assays

Firefly and sea pansy luciferase assays were performed using the dual luciferase assay system. Procedures were performed according to the manufacturer's instructions. Luciferase activities were determined on a Berthold Lumat model LB 9501 (Wildbad, Germany). Firefly luciferase activities (relative light units) were normalized by sea pansy luciferase activities.

For SL2 cells, firefly and β-galactosidase activities were determined as described previously [28]. Firefly luciferase activities were normalized by β-galactosidase activities.

Statistical differences were determined by a two-tailed Student's *t* test.

Production of anti-ZHX2 antibody

A peptide, NH_2-(GC)AKDQLAIAASRHGRTY-CO_2H, was synthesized, conjugated with keyhole limpet haemocyanin, and then immunized against New Zealand White rabbits. Anti-ZHX2 antibody was affinity-purified from antiserum using the SulfoLink kit, according to the manufacturer's instructions.

Immunoprecipitation and Western-blot analysis

Preparation of nuclear extracts from HEK-293 cells was performed as described previously [29]. The nuclear extract (1 mg) was incubated with 5 μg of anti-NF-YA or anti-GST antibody at

4 °C for 2 h. Protein A–Sepharose 4 Fast Flow was then added and followed by an overnight incubation at 4 °C. After washing the beads five times with 2 × PBS, the precipitated proteins were separated by SDS/PAGE (10 % gel) and electroblotted to a PVDF membrane. The membrane was immersed in 5 % (w/v) skimmed milk, 0.1 % (v/v) Tween 20 in PBS for overnight at 4 °C and washed with 0.1 % Tween 20 in PBS. ZHX2 or NF-YA protein was detected and visualized using each specific antibody and the ECL® plus kit. The procedure was performed according to the manufacturer's instructions. Dilutions of anti-ZHX2 and anti-NF-YA antibodies were 1:2000 and 1:4000 respectively.

RESULTS

Molecular cloning of a ZHX1-interacting protein, ZHX2

To analyse the molecular mechanism by which ZHX1 functions as a transcriptional factor, we initially examined the interactions of human ZHX1 with other transcription factors. An entire coding sequence of the human ZHX1 was fused to the DBD of the GAL4 transcription factor. The fusion protein was employed as a bait to screen a rat liver cDNA library using the yeast two-hybrid system. In yeast two-hybrid system, the yeast shows reporter activity only when ZHX1 fused to the GAL4 DBD interacts with GAL4 AD-fusion protein expressed from the cDNA library. Indeed, approx. 15 million independent clones were screened and 16 showed reproducible *HIS3*-, *ADE2*-, *MEL1*-positive properties, and β-galactosidase activity respectively. After a determination of their nucleotide sequences, they were compared with the GenBank® database using the BLAST search program. One of these clones, pAD-L26, exhibited a similarity to the nucleotide sequence of the human KIAA0854 protein. Very interestingly, the deduced amino acid sequence of the KIAA0854 protein has an open reading frame of 837 amino acid residues and contains two Cys_2-His_2-type Znf motifs and five HDs as well as ZHX1, indicating that it also belongs to the Znf class of the homeobox protein superfamily [11]. Hereafter, we refer to the KIAA0854 as ZHX2 and report on its further characterization.

A schematic representation of human ZHX2 and its deduced amino acid sequence compared with human ZHX1 is shown in Figure 1(A). The similarities of the nucleotide sequences in the coding region and amino acid sequences were 45.5 and 41.9 % respectively. The human ZHX2 has a predicted molecular mass of 92 kDa and a pI of 6.42. ZHX2 contains a unique proline-rich region between HD1 and HD2, in addition to two Znf motifs and five HDs. As shown in Figure 1(B), in comparison with human ZHX2, the similarity of the cloned pAD-L26, a partial rat ZHX2 cDNA, was 88 % at the nucleotide level and 93.3 % at the amino acid level respectively. We then determined the tissue distribution of human ZHX2 mRNA by Northern-blot analysis (Figure 2). An approx. 4.4 kb ZHX2 transcript was observed in all the tissues examined, although the intensity of the transcript varied among tissues, indicating that human ZHX2 mRNA is expressed ubiquitously.

Mapping of the minimal heterodimerization domain of ZHX1 with ZHX2

The amino acid sequence of the pAD-L26 corresponds to the amino acid sequence between residues 195 and 358 of human ZHX2 (Figure 1B). We then examined which domain of ZHX1 is required for interaction with ZHX2. An yeast strain SFY526 was transformed with the pDBD, which expresses GAL4 DBD alone, pDBD-L26 or pDBD-ZHX2 (263–322), which encodes

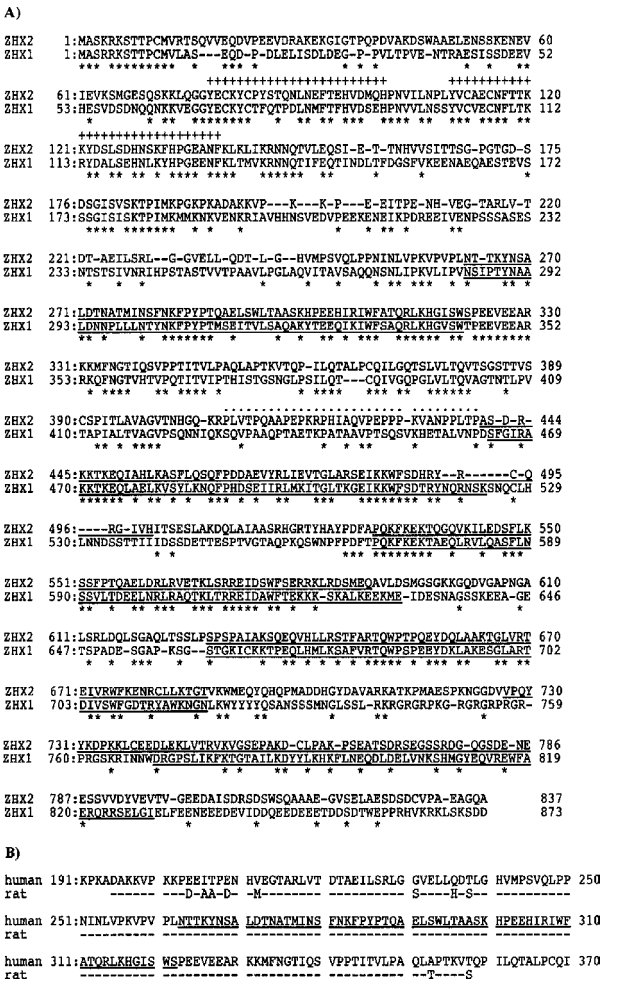

Figure 1 The ZHX2 protein contains two Znf motifs and five HDs as well as ZHX1

(**A**) A comparison of the human ZHX2 amino acid sequence with that of the human ZHX1. The two Znf motifs, proline-rich region and five HDs are indicated by plus signs, dots and underlines respectively. Asterisks indicate the amino acid identity. Dashes indicate a gap where no corresponding amino acid sequence to each protein exists. (**B**) The amino acid sequences of human ZHX2 and pAD-L26 clone (a partial of rat ZHX2 composed of 163 amino acid residues) are compared. The HD is indicated by an underline. Dashes indicate amino acid identity.

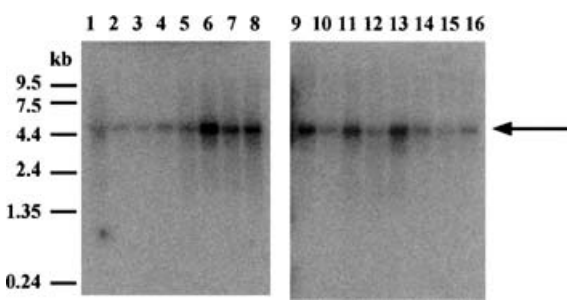

Figure 2 Tissue distribution of human ZHX2 mRNA

A human multiple tissue Northern blot was hybridized with ^{32}P-labelled human ZHX2 cDNA. Prehybridization and hybridization procedures are described in the Experimental section. Each lane contains 2 μg of polyadenylated RNA isolated from the indicated tissues. The molecular-mass markers are shown on the left in kb. Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas; lane 9, spleen; lane 10, thymus; lane 11, prostate; lane 12, testis; lane 13, ovary; lane 14, small intestine; lane 15, colon; lane 16, leucocyte.

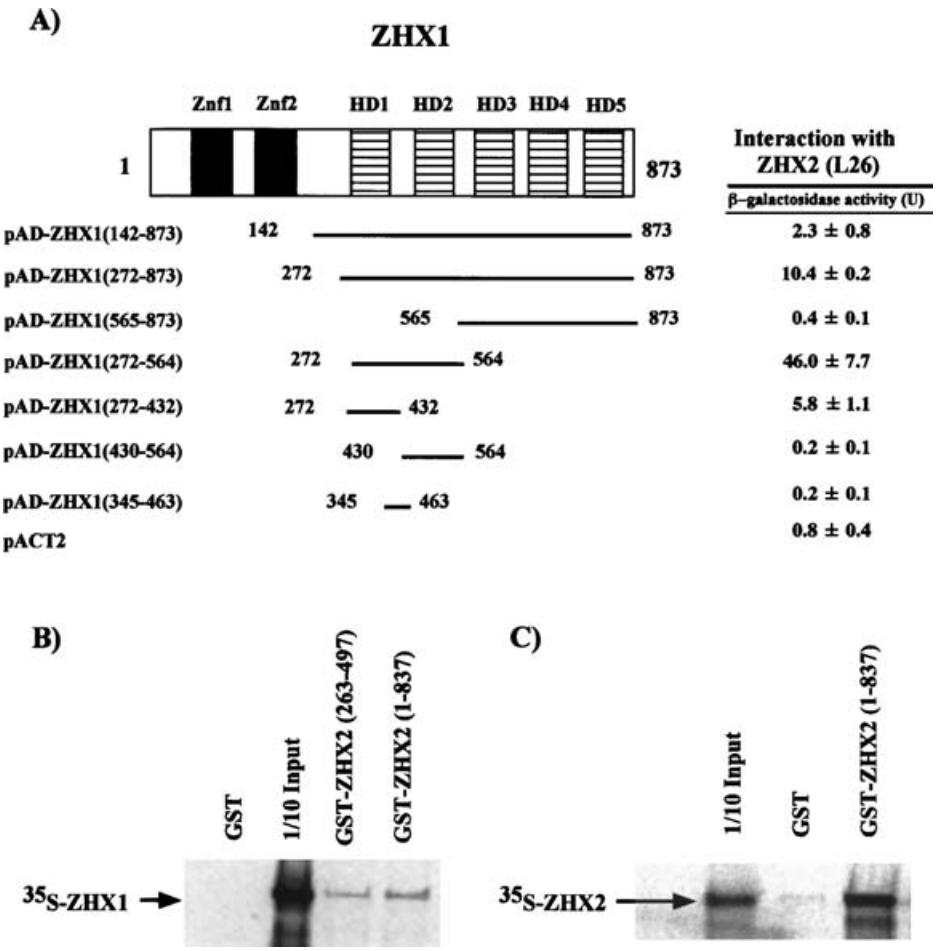

Figure 3 Identification of the minimal hetero and homodimerization domains of ZHX2

(**A**) Mapping of the heterodimerization domain of ZHX1 with ZHX2 using a yeast two-hybrid system. A schematic representation of human ZHX1 is shown at the top left. The GAL4 AD-ZHX1-fusion constructs are depicted on the left. Znf and HD indicate the Znf motif and HD respectively. Each value of β-galactosidase activity represents means $\pm$ S.E.M. from at least three different experiments. (**B**) *In vitro*-translated, ^{35}S-labelled, full-length human ZHX1 was incubated with Sepharose beads containing bound GST alone, a truncated form [GST-ZHX2 (263–497)] or full-length [GST-ZHX2 (1–837)] of the human ZHX2 protein fused to the C-terminal of the GST. The beads were washed thoroughly and the bound protein then eluted and analysed by SDS/PAGE (10 % gel). Interaction signals were detected by autoradiography. The signal in the lane marked '1/10 input' represents 10 % of the protein added to the reactions shown in the other lanes. (**C**) *In vitro*-translated, ^{35}S-labelled, full-length human ZHX2 was incubated with Sepharose beads containing bound GST alone, full-length of the human ZHX2 protein [GST-ZHX2 (1–837)] fused to the C-terminal of the GST.

the amino acid sequence between residues 263 and 322 of human ZHX2 fused to the GAL4 DBD. The three yeast strains were used as the reporter yeasts. The pACT2, which expresses GAL4 AD alone or some plasmids, which encodes various truncated forms of ZHX1 fused to the GAL4 AD, were employed as the prey plasmids. When a reporter yeast harbouring the pDBD-L26 was transformed with one among pAD-ZHX1 (142–873), pAD-ZHX1 (272–873), pAD-ZHX1 (272–564) or pAD-ZHX1 (272–432), they showed high β-galactosidase activities. However, the yeast was transformed with one among pACT2, pAD-ZHX1 (565–873), pAD-ZHX1 (430–564) or pAD-ZHX1 (345–463), these yeasts also showed low β-galactosidase activities (Figure 3A). In contrast, when a reporter yeast harbouring the pDBD or pDBD-ZHX2 (263–322) was transformed with these prey plasmids, they showed low β-galactosidase activities (results not shown). These results indicate that ZHX1 and ZHX2 heterodimerize through a region containing each of the HD1.

We then used GST pull-down assays *in vitro* to examine the specific interaction between ZHX1 and ZHX2. We employed three plasmids, the pGEX-5X-1, which expresses GST alone, pGST-ZHX2 (263–497), which encodes the amino acid sequence between 263 and 497 of human ZHX2 fused to the GST and pGST-ZHX2 (1–837), which expresses the full-length of human ZHX2 protein fused to the GST respectively. These proteins were expressed in *Escherichia coli* and immobilized on glutathione–Sepharose beads. In the GST pull-down assays, only when the *in vitro*-translated, ^{35}S-labelled, full-length of human ZHX1 interacts with GST-fusion protein, was the interaction signal detected as a band corresponding to the size of ZHX1 by autoradiography. Indeed, ZHX1 was found to bind to GST-ZHX2 (263–497) and GST-ZHX2 (1–837) but not to GST alone (Figure 3B). In contrast, an unprogrammed reticulocyte lysate failed to bind to any protein (results not shown). These results indicate that ZHX1 is capable of forming a heterodimer with ZHX2 both *in vivo* and *in vitro*.

Mapping of the minimal homodimerization domain of ZHX2

ZHX1 forms a homodimer [10]. We then investigated homodimer formation for ZHX2 using the yeast two-hybrid system and GST pull-down assays.

Table 1 Analysis and determination of the homodimerization domain of ZHX2 using a yeast two-hybrid system

Each value represents means ± S.E.M. of β-galactosidase activities (units) from at least three different experiments. n.d., not determined.

	β-Galactosidase activitiy (units)			
	pDBD	pDBD-ZHX2 (263–322)	pDBD-L26	pDBD-ZHX2 (1–837)
pACT2	0.14 ± 0.05	0.06 ± 0.01	0.22 ± 0.05	0.21 ± 0.06
pAD-ZHX2 (263–322)	0.13 ± 0.02	0.13 ± 0.03	0.19 ± 0.12	n.d.
pAD-L26	0.11 ± 0.06	0.09 ± 0.04	1.51 ± 0.14	n.d.
pAD-ZHX2 (1–837)	0.32 ± 0.13	0.14 ± 0.02	2.62 ± 0.31	1.03 ± 0.14

We first performed a yeast two-hybrid assay. A reporter yeast strain harbouring pDBD-ZHX2 (1–837), which expresses an entire coding sequence of human ZHX2 fused to the GAL4 DBD, was used. When the pAD-ZHX2 (1–837), which expresses full-length human ZHX2 fused to the GAL4 AD, was transformed into the reporter yeast, β-galactosidase activity was determined (Table 1). We next prepared three other SFY526 yeast strains harbouring the pDBD, pDBD-ZHX2 (263–322) or pDBD-L26, as the reporter yeasts. We prepared four prey plasmids, the pACT2, pAD-ZHX2 (263–322), pAD-L26 and pAD-ZHX2 (1–837). These plasmids were transformed into the reporter yeasts and β-galactosidase activity was determined in each case (Table 1). When the reporter yeast harbouring the pDBD or pDBD-ZHX2 (263–322) was transformed with the plasmids, they showed very low β-galactosidase activities. In addition, when the reporter yeast harbouring the pDBD-L26 was transformed with the pACT2 or pAD-ZHX2 (263–322), it showed a very low β-galactosidase activity as well. In contrast, the yeast transformed with the pAD-L26 or pAD-ZHX2 (1–837) expressed higher β-galactosidase activities. These results indicate that ZHX2 is capable of forming a homodimer via a region corresponding to the amino acid sequence between residues 195 and 358.

We then performed GST pull-down assays to examine the homodimerization of ZHX2 *in vitro*. We employed two plasmids, the pGEX-5X-1 and pGST-ZHX2 (1–837). These proteins were expressed in *E. coli* and immobilized on glutathione–Sepharose beads. The *in vitro*-translated, ^{35}S-labelled, full-length human ZHX2 was found to bind to GST-ZHX2 (1–837) but not to GST alone (Figure 3C). In contrast, an unprogrammed reticulocyte lysate failed to bind to any protein (results not shown).

These results indicate that ZHX2 is capable of forming a homodimer both *in vivo* and *in vitro*.

ZHX2 also interacts with the AD of NF-YA

Human ZHX1 was cloned originally as a protein that interacts with NF-YA [8]. We then examined the interaction of ZHX2 with NF-YA using the yeast two-hybrid system. We used two-reporter yeast strains, which are transformed with pDBD or pYA1–269 respectively. The pYA1–269 expresses the AD of NF-YA fused to the GAL4 DBD. Four plasmids, pACT2, pAD-L26, pAD-ZHX2 (263–497) and pAD-ZHX2 (1–837) were transformed into the reporter yeast strains and their β-galactosidase activities were determined. When a reporter yeast harbouring the pDBD is transformed with these plasmids, the resulting β-galactosidase activities were quite low (results not shown). In addition, when a reporter yeast harbouring the pYA1–269 was transformed with pACT2 or pAD-L26, the resulting β-galactosidase activities were also low. However, when the yeast was transformed with the pAD-ZHX2 (263–497) or pAD-ZHX2 (1–837), high levels of β-galactosidase activities were found. These results indicate that ZHX2 interacts with the AD of NF-YA, and that the amino acid sequence between residues 263 and 497 of ZHX2 is essential for this interaction.

We next identified the minimal interaction domain of NF-YA with ZHX2 using the yeast two-hybrid system. As shown in Figure 4(B), the AD of NF-YA consists of a glutamine- and a serine/threonine-rich domains [8]. An SFY526 yeast strain harbouring pAD-ZHX2 (263–497) was used as a reporter yeast. Various plasmids that express truncated forms of NF-YA fused to GAL4 DBD were transformed in yeast and their β-galactosidase activities determined. As a result, yeasts harbouring pYA1–269 or pYA141–269, both containing the amino acid sequence between residues 141 and 269 of NF-YA, showed a high level of β-galactosidase activity. When a reporter yeast harbouring pACT2 was transformed with plasmids that express the GAL4 DBD–NF-YA-fusion protein, yeast showed marginal β-galactosidase activities (results not shown). These results indicate that a serine/threonine-rich AD of NF-YA represents a minimal interaction domain with ZHX2.

Furthermore, to verify the interaction of ZHX2 and NF-YA in mammalian cells, a nuclear extract from HEK-293 cells was immunoprecipitated with the anti-NF-YA antibody. As shown in Figure 4(C), both ZHX2 and NF-YA were detected in the immunoprecipitated complex using Western-blot analysis. In contrast, in the case of immunoprecipitation with anti-GST antibody, neither ZHX2 nor NF-YA was detected. This result also shows that ZHX2 interacts intrinsically with NF-YA in HEK-293 cells.

ZHX2 is a transcriptional repressor

We determined the transcriptional role of ZHX2 using a mammalian one-hybrid system. The 5 × GAL4-pGL3 control plasmid, in which five copies of the GAL4-binding site had been inserted upstream of the simian virus 40 (SV40) promoter of the pGL3 control, was employed as a reporter plasmid [21]. Two effector plasmids, the pSG424, which expresses GAL4 DBD alone, and pGAL4-ZHX2 (1–837), which expresses the entire coding region of human ZHX2 fused to the C-terminus of the GAL4 DBD, were prepared. In the mammalian one-hybrid system, the GAL4 DBD–ZHX2-fusion protein binds to the GAL4-binding site, and then affects transcriptional activity of the luciferase reporter gene. When the GAL4–ZHX2-fusion protein is a transcriptional activator, firefly luciferase activity is increased. In contrast, when the GAL4–ZHX2-fusion protein is a transcriptional repressor, luciferase activity is decreased. As shown in Figure 5, when 5 × GAL4-pGL3 control and various amounts of pGAL4-ZHX2 (1–837) were co-transfected into HEK-293 cells, luciferase activity decreased in a dose-dependent manner. Maximal inhibition was obtained with 50 ng of pGAL4-ZHX1 (1–837). In contrast, when pGL3 control lacking five copies of GAL4-binding sites was transfected with pSG424 or pGAL4-ZHX2 (1–837), luciferase activities remained unchanged (Figure 5). These results show that the GAL4–ZHX2-fusion protein decreases luciferase activity in a GAL4-binding site-dependent manner, indicating that ZHX2 acts as a transcriptional repressor.

Furthermore, to determine the minimal repressor domain of ZHX2, the 5 × GAL4-pGL3 control was then transfected with three plasmids, the pGAL4-ZHX2 (1–262), pGAL4-ZHX2 (263–497) or pGAL4-ZHX2 (441–783) (Figure 6). Only the pGAL4-ZHX2 (263–497), which expresses the amino acid sequence between residues 263 and 497, led to a 42 % decrease in luciferase

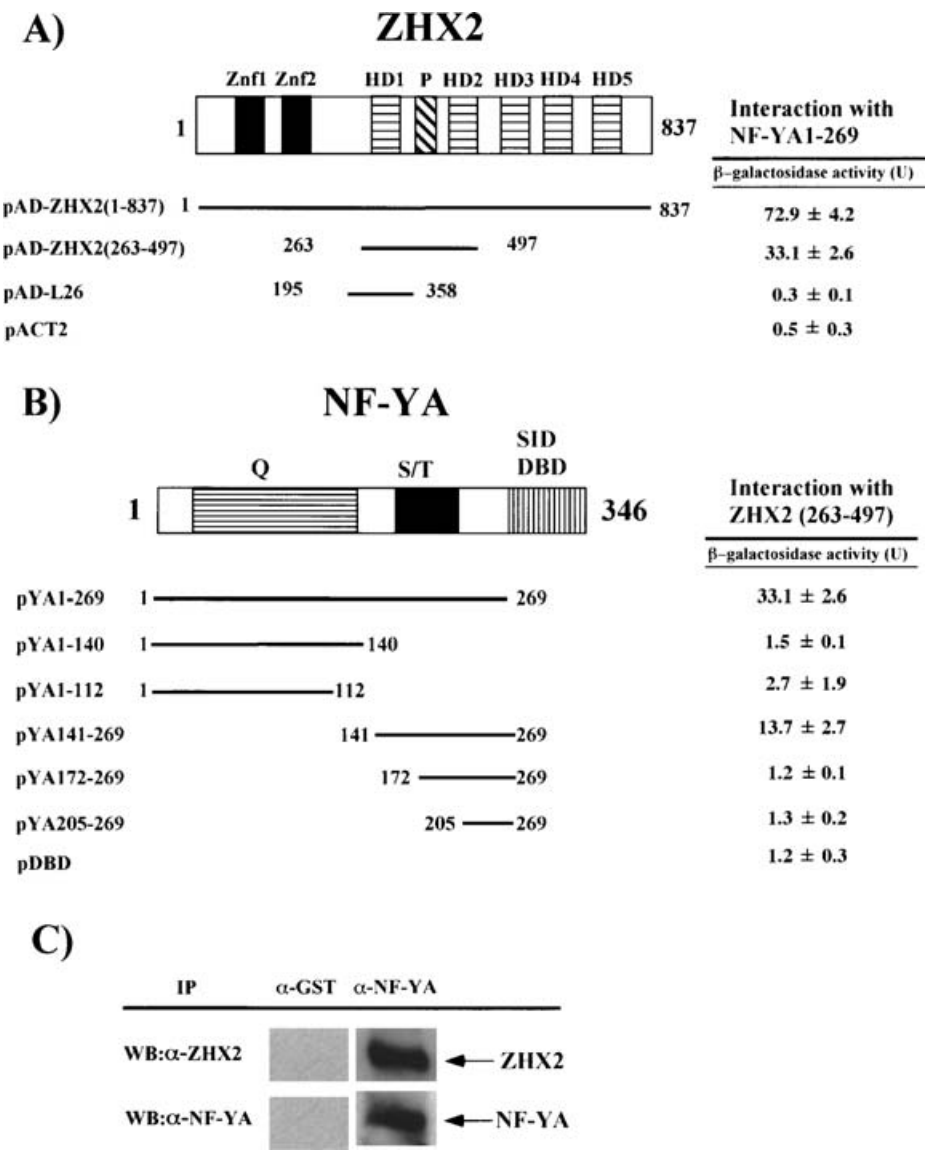

Figure 4 Determination of the interaction domains of ZHX2 and NF-YA

(**A**) A schematic representation of human ZHX2 at the top left. GAL4 AD-ZHX2-fusion constructs are depicted on the left. Znf and HD indicate the Znf motif and HD respectively. Each value of β-galactosidase activity represents means $\pm$ S.E.M. from at least three different experiments. (**B**) A schematic diagram of NF-YA and its deletion mutants fused GAL4 DBD is illustrated on the left. Q and S/T refer to a glutamine-rich and a serine/threonine-rich region respectively. SID stands for subunit-interaction domain. Each value of β-galactosidase activity represents means $\pm$ S.E.M. from at least three different experiments. (**C**) Immunoprecipitation of a nuclear extract (1 mg of protein) from HEK-293 cells was performed using the anti-NF-YA or anti-GST polyclonal antibody (5 μg). The immunoprecipitated complex was separated by SDS/PAGE (10 % gel), and was detected by Western-blotting assay using anti-ZHX2 or anti-NF-YA antibody with ECL® Plus Western-blotting reagent pack. IP, immunoprecipitation assay; WB, Western-blotting assay; α-GST, anti-GST antibody; α-NF-YA, anti-NF-YA antibody; α-ZHX2, anti-ZHX2 antibody.

activity. In a more detailed analysis, we prepared the effector plasmids, pGAL4-ZHX2 (263–446), pGAL4-ZHX2 (263–322) or pGAL4-ZHX2 (317–497). When pGAL4-ZHX2 (263–446), but not pGAL4-ZHX2 (263–322) or pGAL4-ZHX2 (317–497), was transfected with the reporter plasmid, luciferase activity was decreased (Figure 6; results not shown). These results show that the amino acid sequence between residues 263 and 446 of ZHX2 is essential for repressor activity.

To examine the issue of whether repressor activity of ZHX2 requires formation of heterodimer with ZHX1, we prepared pCMV-ZHX1 (272–432) that expresses the minimal domain of ZHX1 required for heterodimerization with ZHX2 and has no repressor activity [12]. When various amounts of the pCMV-ZHX1 (272–432) were co-transfected with 5 $\times$ GAL4-pGL3 control and either pSG424 or pGAL4-ZHX2 (1–837) into HEK-293 cells, luciferase activities were unchanged (results not shown). These results suggest that the repressor activity of ZHX2 does not depend on heterodimerization of ZHX1 with ZHX2.

Determination of subcellular localization of ZHX2 and mapping of nuclear localization signal (NLS)

To examine the subcellular localization of the ZHX2 protein, we employed the GFP-ZHX2-fusion protein expression system. Various truncated forms of ZHX2 fused to the GFP were prepared. These plasmids were transfected into HEK-293 cells and subcellular localization of GFP-fusion proteins was observed.

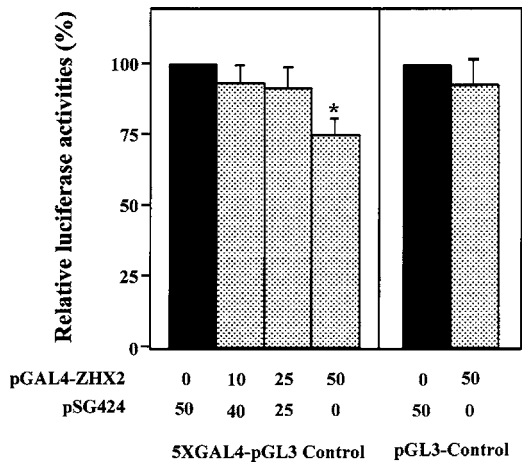

Figure 5 ZHX2 is a transcriptional repressor

HEK-293 cells were co-transfected with 2 ng of pRL-CMV, 50 ng of SV40 promoter-directed expression vector (shown on the column) and 200 ng of 5 × GAL4-pGL3 control or pGL3 control reporter plasmid. The pSG424 and pGAL4-ZHX2 (1–837) express GAL4 DBD alone and the entire coding sequence of human ZHX2 fused to the GAL4 DBD respectively. The cells were harvested 48 h after transfection and both firefly and sea pansy luciferase activities were determined. Firefly luciferase activities were normalized by sea pansy luciferase activities in all experiments. A value of 100 % was assigned to the promoter activity of the reporter plasmid in the presence of 50 ng of pSG424 respectively. Each column and bar represents means ± S.E.M. from at least five different transfection experiments. $*P < 0.01$ by Student's *t* test.

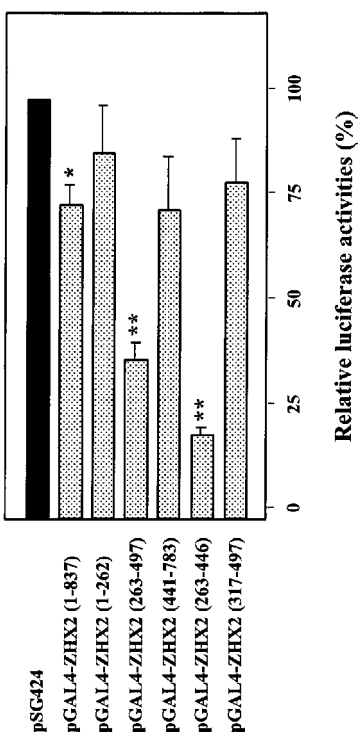

Figure 6 Determination of the minimal repressor domain of ZHX2

The pSG424 and various pGAL4-ZHX2 construct express GAL4 DBD alone and various deletion mutants of human ZHX2 fused to the GAL4 DBD respectively. Conditions are the same as described in the legend to Figure 5. Each column and error bar represent means ± S.E.M. from at least five different transfection experiments. $*P \times 0.05$ and $**P \times 0.001$ by Student's *t* test.

The pEGFP-C1E1 encoding GFP protein alone was observed in the whole cells (Figure 7). In contrast, GFP-ZHX2 (1–837), in which full-length ZHX2 was fused to the C-terminus of GFP, was localized in the nuclei. To determine the NLS of ZHX2, various plasmids were transfected. Only when three plasmids pGFP-ZHX2 (263–497), pGFP-ZHX2 (263–446) and pGFP-ZHX2 (317–446) were transfected, nuclear localization of ZHX2 was observed. However, when pGFP-ZHX2 (317–407) or pGFP-ZHX2 (408–440) was transfected, both proteins failed to localize in the nuclei. These results show that ZHX2 is capable of localizing in the nuclei as a GFP-fusion protein, and that the NLS of ZHX2 is the amino acid sequence between residues 317 and 446.

ZHX2 represses promoter activity of the *cdc25C* gene stimulated by NF-Y in SL2 cells

To examine the function of ZHX2 in NF-Y-regulated gene expression, we performed a promoter assay using SL2 cells. This cell line is devoid of endogenous NF-Y [26]. We prepared a luciferase reporter plasmid, which contains the nucleotide sequence between −172 and +10 of the human *cdc25C* gene promoter [13]. It has been reported that the human *cdc25C* promoter contains three NF-Y-binding sequences and is transactivated by NF-Y [13]. In fact, co-transfection of NF-Y expression plasmids into SL2 cells strongly enhanced the promoter activity of pCDC25/Luc, and the luciferase activities increased by approx. 165-fold (Figure 8). When various amounts of pPac-ZHX2, which encodes an entire coding sequence of human ZHX2, were co-transfected into SL2 cells, the luciferase activities stimulated by NF-Y led to a decrease in a dose-dependent manner. These results indicate that ZHX2 represses the promoter activity of the *cdc25C* gene stimulated by NF-Y.

DISCUSSION

We cloned a novel transcriptional repressor ZHX2 as a ZHX1-interacting protein and mapped the functional domains. ZHX2 is a transcriptional repressor, contains two Znf motifs and five HDs, forms a homodimer, interacts with the AD of NF-YA and is localized in the nuclei. In addition, ZHX2 mRNA is expressed ubiquitously. These findings indicate that ZHX2 shares many properties with ZHX1. Thus we conclude that ZHX1 and ZHX2 are members of the same family, namely the ZHX family.

The two Znf motifs and five HDs were conserved between ZHX1 and ZHX2. The similarity of the entire amino acid sequences of ZHX1 and ZHX2 was 41.9 %. Similarities in the amino acid sequences of Znf1, Znf2, HD1, HD2, HD3, HD4 or HD5 between ZHX1 and ZHX2 were 62.5, 73.3, 41.9, 49.2, 45, 47.5 and 21 % respectively. HD5 showed a lower similarity than the other domains. A unique proline-rich region is located in the amino acid sequence between residues 408 and 440 of ZHX2 (Figure 1A). Generally, the Znf motif, HD, basic and acidic regions, and a proline-rich region are related to functional aspects of the transcription factor. It has been reported that the Znf motif is required for binding to the cognate DNA sequence through a Zn^{2+} ion, the HD of which consists of a 60 amino acid residues, the DBD, the basic region is the DBD or NLS, and the acidic and proline-rich regions are involved in transcriptional activity [11,21,30–33].

ZHX2 not only forms a heterodimer with ZHX1 but also forms a homodimer. The amino acid sequence between residues 195 and 358 of ZHX2 is necessary and sufficient for dimerization (Table 1 and Figure 3A). The homo- and heterodimerization of ZHX1 requires the amino acid sequence between residues 272

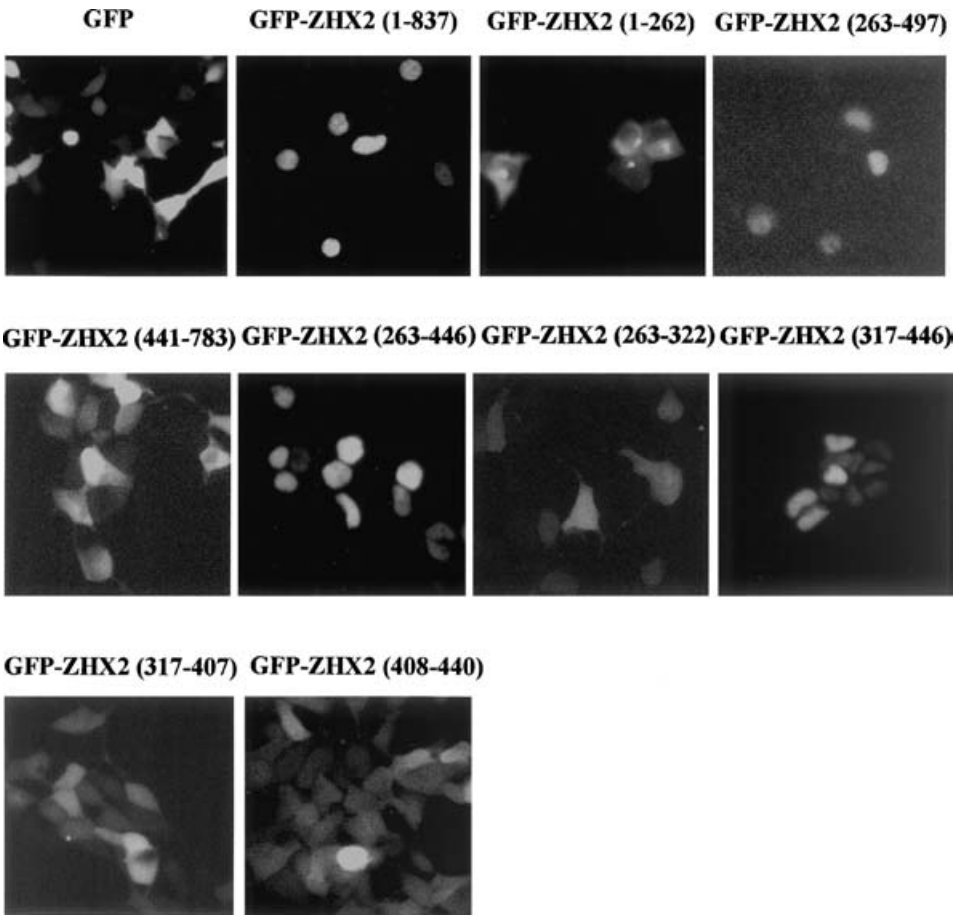

Figure 7 Subcellular localization of ZHX2 in HEK-293 cells and a determination of the NLS

Expression plasmids (300 ng) that encode GFP alone or various truncated ZHX2 proteins fused to the C-terminus of GFP, were transfected into HEK-293 cells. Subcellular localization of GFP-fusion protein was observed 72 h after transfection.

and 432 [12]. Although these regions include the HD1, the HD1 alone in both proteins failed to form the dimers. Therefore a more extensive region containing the HD1 is required for dimerization. Both ZHX1 and ZHX2 interact with the AD of NF-YA. ZHX1 interacts with a glutamine-rich AD of NF-YA via the amino acid sequence between residues 272 and 564 of ZHX1 [8]. For ZHX2, the protein interacts with a serine/threonine-rich AD of NF-YA via the region between 263 and 497 of ZHX2 (Figures 4A and 4B). These regions of both ZHX proteins include the HD1 to HD2 region (Figures 3A and 4A). Therefore the dimerization domain of ZHX proteins also overlaps with the interaction domain with the AD of NF-YA.

ZHX2 is a transcriptional repressor (Figures 5 and 6). The minimal repressor domain of ZHX2 is mapped to the amino acid sequence between residues 263 and 446, which contains the HD1 to a proline-rich region, and overlaps with the dimerization domain. The repressor domain of ZHX1 is localized in the C-terminal acidic region, and homodimerization is required for the full repressor activity of ZHX1 [12]. Furthermore, we cloned successfully another member of the ZHX family, ZHX3, which is also a transcriptional repressor, and the repressor activity depends on heterodimerization with ZHX1 [23]. In contrast, the repressor activity of ZHX2 was not affected by overexpression of the dimerization domain of ZHX1. This, therefore, suggests that ZHX2 has inherent repressor activity or achieves it by an interaction(s) with other transcriptional regulators different from ZHX1. The p53 protein contains a proline-rich region that consists of 15 amino acid residues and also contains two Pro-Xaa_2-Pro motifs. It has been reported that this region is necessary for the transcriptional repression of p53 [34,35] and that it interacts with mSin3A [36]. mSin3A is an ubiquitous nuclear co-repressor that is associated with a number of other transcriptional repressors [37]. Since ZHX2 contains three Pro-Xaa_2-Pro motifs in the proline-rich region, it may associate with mSin3A for repressor activity.

When the entire coding region of ZHX2 was fused to the GFP, it became localized in the nuclei (Figure 7). In many other proteins, it has been reported that the NLS is mapped to a cluster of basic amino acid residues [38]. This region is associated with the nuclear importing proteins such as importin α and is then translocated from the cytoplasm to the nuclei [31]. In contrast, ZHX2 may associate with other molecules for translocation to the nuclei, since the NLS of ZHX2 is mapped to the amino acid sequence between residues 317 and 446, which include the proline-rich region but not the basic region.

The size of ZHX2 mRNA was determined to be approx. 4.4 kb, as determined by Northern-blot analysis (Figure 2). Although ZHX2 mRNA is expressed ubiquitously, it was expressed more highly in ovary, prostate, spleen, skeletal muscle and pancreas. Two classes of co-factor, a co-activator and a co-repressor, such as p300, P/CAF and mSin3A, are also expressed ubiquitously.

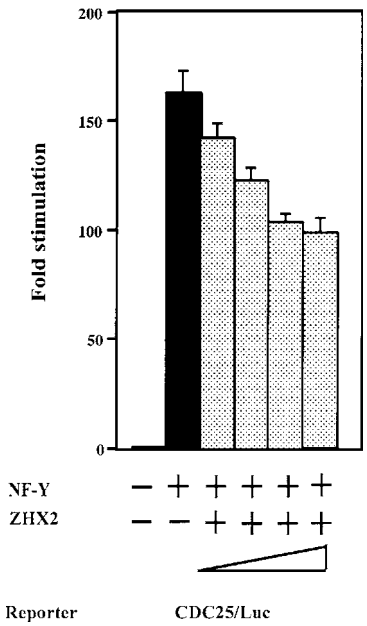

Figure 8 ZHX2 represses promoter activity of the *cdc25C* gene stimulated by NF-Y in *Drosophila* SL2 cells

pCDC25/Luc reporter plasmid (2 μg) was co-transfected with 100 ng of pPac-β-Gal, 25 ng of pPac or 25 ng of pPacNF-YA, pPacNF-YB and pPacNF-YC with or without various amounts of pPac-ZHX2 into SL2 cells. Total amount of DNA (2.325 μg) was adjusted by the addition of the pPac plasmid. Each column and error bar represents means $\pm$ S.E.M. from at least four different transfection experiments. Results are presented as fold stimulation where the value of luciferase activity normalized to β-galactosidase activity for the reporter alone is set at 1.0.

They play important transcriptional roles in a gene-specific manner via their interactions with cell-type-specific or ligand-dependent transcription factors [1,2]. Generally, transcription regulatory regions interact with co-factors to function as transcriptional activators or repressors [1,2]. It has been reported that NF-Y associates with p300 and P/CAF [4] and, in particular, P/CAF, a transcriptional co-activator with histone acetyltransferase activity, interacts with the NF-YA to form a transcriptionally active NF-Y complex [4]. Therefore it is likely that combinations of interactions among ZHX1, ZHX2 and NF-YA affect the transcriptional activity of NF-Y. As shown in Figure 4(C), ZHX2 intrinsically interacts with NF-YA in the nuclei of HEK-293 cells, suggesting that ZHX2 participates in the regulation of a number of NF-Y-regulatable genes. Indeed, ZHX2 decreased the *cdc25C* promoter activity stimulated by NF-Y in a dose-dependent manner (Figure 8). It has been reported that the cdc25C protein regulates cell-cycle progression into the M phase and the *cdc25C* gene transcription occurs late in the S/G_2 phase [39]. Therefore ZHX2 may be involved in cell growth and differentiation in a variety of tissues.

Recently, we reported that the mouse *ZHX1* gene is approx. 29 kb in length, exists as one copy in a haploid mouse genome and consists of five exons. The entire coding sequence of ZHX1 is contained in exon 4. Transcription of the *ZHX1* gene is synergistically stimulated by PEA3 and YY1 respectively [40]. We also cloned another member of the ZHX family, ZHX3 [23]. Interestingly, when the human *ZHX1*, *ZHX2* and *ZHX3* genes were searched, using the database compiled by the human genome project, both *ZHX1* and *ZHX2* genes were found to be located in chromosome 8q, and the distance between them was only 396 kb with a reverse orientation of transcription. (The HUGO Gene Nomenclature Committee has approved ZHX2 as the abbreviation for zinc-fingers and homeoboxes 2.) The *ZHX3* gene is located on a different chromosome. These findings suggest that the *ZHX1* and *ZHX2* genes may have been produced by gene duplication of an ancestral gene.

In summary, we report on the molecular cloning and characterization of a novel transcriptional repressor ZHX2, a new protein of the ZHX family with two Znf motifs and five HDs. It is possible that members of the ZHX family exist in a more organized transcription network and regulate gene transcription via interaction with NF-YA. Further studies will be required to understand the biological role of these ZHX proteins, and to search for other possible members of this family.

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