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Rat zinc-fingers and homeoboxes 1 (ZHX1), a nuclear factor-YA-interacting nuclear protein, forms a homodimer

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Abstract

Zinc-fingers and homeoboxes 1 (ZHX1) is a protein which interacts with the activation domain of the A subunit of nuclear factor-Y. To analyze the physiological role(s) of ZHX1, we searched ZHX1-interacting protein(s) using a yeast two-hybrid system. The rat counterpart of ZHX1 cDNAs was cloned from an ovarian granulosa cell complementary DNA (cDNA) library, indicating that ZHX1 is able to form a homodimer. An analysis of the nucleotide sequence and its deduced amino acid sequence show that rat ZHX1 consists of 873 amino acid residues. Northern blot analysis shows that ZHX1 messenger RNA is expressed ubiquitously and that the level in the ovary are not regulated by gonadotropins. Furthermore, transfection experiments with green fluorescence protein (GFP) expression vectors into human embryonic kidney HEK293 cells reveal that full-length ZHX1 fused to the GFP is localized in the nuclei. Thus, we report on the molecular cloning, expression and characterization of full-length rat ZHX1 cDNA.

Keywords: Yeast two-hybrid; Transcription factor; Homeobox; Zinc-finger motif; Dimerization; Nuclear localization

1. Introduction

Granulosa cells play important roles in ovarian function including follicular maturation and ovulation (Richards, 2001). Many granulosa cell genes express proteins in a cell type-specific manner, and some of these are also transcriptionally regulated by gonadotropins, such as follicle stimulating hormone and luteinizing hormone. Generally, gene transcription is positively or negatively regulated by transcription factors which bind to the regulatory region within the promoter of the gene. Cell type-specific transcription factors and ubiquitous transcription factors are known to exist (Fry and Farnham, 1999). Unique combinations of these factors on the promoter serves to determine gene-specific transcription. Most transcription factors not only bind to the specific *cis*-acting element but also interact with one another. In addition to these DNA-binding proteins, DNA-non-binding proteins also regulate the transcription of the target genes. These factors, referred to as the co-activators or co-repressors, act by bridging or interfering with interactions among DNA-binding proteins and basic transcription machinery (Hu and Lazar, 2000; Vo and Goodman, 2001).

Nuclear factor-Y (NF-Y), a ubiquitous transcription factor, binds to an inverted CCAAT nucleotide sequence (Y box, 5′-ATTGG-3′) and stimulates the transcription of a number of genes (Mantovani, 1999). NF-Y is thought to be essential for cell proliferation stimulated by estradiol in MCF-7 cells through the activation of various cell cycle related genes (Wang et al., 1999). NF-Y consists of three subunits, YA, YB, and YC, which also interact with other transcription factors (Mantovani, 1999; Yamada et al., 2000). We previously cloned human zinc-fingers and homeoboxes 1 (ZHX1) and serum response factor as proteins

Abbreviations: NF-Y, nuclear factor-Y; ZHX1, zinc-fingers and homeoboxes 1; AD, activation domain; Znf, zinc-finger; HD, homeodomain; kb, kilobases; DES, diethylstilbestrol; GST, glutathione-*S*-transferase; DBD, DNA-binding domain; GFP, green fluorescence protein; RACE, rapid amplification of the cDNA ends; USF, upstream stimulatory factor; NLS, nuclear localization signal

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which interact with the activation domain (AD) of NF-YA (Yamada et al., 1999a,b). Mouse ZHX1 was independently reported as an antigen which is recognized by the B92 monoclonal antibody produced by immunization with a cell lysate of 14F1.1 endothelial-adipose stromal cells (Barthelemy et al., 1996). Both human and mouse ZHX1 consist of 873 amino acid residues and contains two Cys_2-His_2-type zinc-fingers (Znf) and five homeodomains (HDs) (Barthelemy et al., 1996; Yamada et al., 1999b). It belongs to the Znf class of the homeobox protein superfamily (Gehring et al., 1994). The amino acid sequence between 272 and 564 that contains the HD1 through HD2 region of the human ZHX1 is required for interaction with a glutamine-rich AD of NF-YA (Yamada et al., 1999a). Northern blot analysis of poly(A)$^+$-RNA isolated from various human tissues has shown that two major ZHX1 transcripts comprised of about 4.5 and 5 kilobases (kb) are expressed ubiquitously (Yamada et al., 1999b). The human *ZHX1* gene is located on chromosome 8q, between markers CHLC.GATA50B06 and CHLC.GATA7G07 (Yamada et al., 1999b).

To address the issue of whether ZHX1 interacts with protein(s) other than NF-YA in rat granulosa cells, we employed a yeast two-hybrid system. Here, we report on the molecular cloning and characterization of full-length rat ZHX1 cDNA.

2. Materials and methods

2.1. Hormones and materials

Diethylstilbestrol (DES) was purchased from Sigma Chemical Co. (St. Louis, MO) and hCG was obtained from Sankyo Co., Ltd. (Tokyo, Japan). The eCG was obtained from Teikokuzouki, Inc. (Tokyo, Japan). The yeast two-hybrid system, rat brain marathon complementary DNA (cDNA), Advantage 2 polymerase chain reaction (PCR) kit, rat Multiple Tissue Northern blot, ExpressHyb hybridization solution and pEGFP-C1 vector were purchased from CLONTECH (Palo Alto, CA). The ExTaq DNA polymerase and BcaBest DNA labeling kit were obtained from Takara BIOMEDICALS (Kyoto, Japan). The pGEM-T Easy vector and T7 TNT Quick-coupled transcription/translation system were purchased from Promega (Madison, WI). The Big Dye terminator FS cycle sequencing kit was purchased from Applied Biosystems Japan (Tokyo, Japan). The pGEX-5X-1 vector, Glutathione-Sepharose 4B, and ^{35}S-methionine (37 TBq/mmol) were purchased from Amersham Pharmacia Biotech (Cleveland, OH). The TOPP3 cells were obtained from Stratagene (La Jolla, CA). α-^{32}P dCTP (111 TBq/mmol) was purchased from NEN Life Science Products (Wilmington, DE). The TRIZOL reagent, Superscript II, and LIPOFECTAMINE PLUS were purchased from Invitrogen (Groningen, Netherlands). The QIAGEN plasmid kit was purchased from QIAGEN (Hilden, Germany). The FuGENE 6 transfection reagent was purchased from Roche Molecular Biochemicals Mannheim (Indianapolis, IN). HEK293 cells, a human embryonic kidney cell line, were purchased from the American Type Culture Collection (Manassas, VA).

2.2. Animals and treatment

Kwl:Wistar immature female rats (21 days old) were used in this study. The rats were treated with 2 mg DES in 0.1 ml sesame oil once daily for 4 days for the preparation of granulosa cells. For the in vivo study, a 30 IU of eCG alone, or 50 IU of hCG after eCG treatment for 48 h were administrated and ovaries were removed at the indicated times. At all times, the animals were treated following NIH guidelines.

2.3. Plasmids

The pBSII-hZHX1E/X, pEGFP-C1E1, pGEM-T-hZHX1E, and pGST-ZHX1E/X plasmids has been described previously (Inazu et al., 1999; Yamada et al., 1999a,b). A 1.9-kb *Eco*RI/*Bam*HI fragment was isolated from the pBSII-hZHX1E/X and subcloned into the *Eco*RI/*Bam*HI sites of the pGBKT7 and pEGFP-C1E1 plasmids to produce pGBKT7-ZHX1EB and pEGFP-ZHX1EB, respectively. A PCR was carried out using the pGEM-T-hZHX1E as a template and 1ZHX1, 5′-CCGGGAATTCATGGCAAGCAGGCGAAAATC-3′, and 272ZHX1, 5′-GGGATTAAGACTTTGGGAATC-3′, as primers. PCR conditions have been described previously except for the use of the ExTaq DNA polymerase (Yamada et al., 1999a). The product was subcloned into the pGEM-T Easy vector to produce pGEM-T Easy-ZHX1 (1–271). A 0.9-kb *Eco*RI fragment from the pGEM-T Easy-ZHX1 (1–271) was subcloned into the *Eco*RI site of the pGST-ZHX1E/X, pGBKT7-ZHX1EB and pEGFP-ZHX1EB to obtain pGST-ZHX1(1–873), pGBKT7-ZHX1 (1–873) and pEGFP-ZHX1 (1–873), respectively. Each plasmid expresses the entire coding region of the human ZHX1 fused to glutathione-*S*-transferase (GST), to DNA-binding domain (DBD) of the yeast GAL4 transcription factor, or to the green fluorescence protein (GFP). The nucleotide sequences of all plasmids were determined.

2.4. Yeast two-hybrid system and library screening

AH109 yeast cells were transformed with the pGBKT7-ZHX1 (1–873) plasmid. A TE/LiAc-based high efficiency transformation method was used for the yeast transformation (Yamada et al., 1998). This yeast strain was used as a bait to screen a cDNA library. The construction of the rat granulosa cell cDNA library has been described previously (Yamada et al., 2001). Approximately eleven million independent clones of the granulosa cell cDNA library were plated on histidine-, tryptophan-, leucine-, and adenine-free synthetic dextrose (SD-His-Trp-Leu-Ade) plates supplemented with 4 mM 3-aminotriazole and X-α-gal.

One hundred and nine positive clones were obtained from the primary transformants.

2.5. Liquid β-galactosidase assays

The yeast strain SFY526 that contains a quantifiable *lacZ* reporter, and either the pGBKT7 or pGBKT7-ZHX1 (1–873) plasmids, was transformed with plasmids isolated from positive clones in primary screening or the parent vector, pACT2. In this second screening, 25 clones from the granulosa cell cDNA library specifically exhibited reproducible high β-galactosidase activity, respectively. Quantitative β-galactosidase assays, using *o*-nitrophenyl-β-D-galactoside, were carried out on permeabilized cells, as described previously (Ausubel et al., 1994; Yamada et al., 1999a,b).

2.6. Nucleic acid sequencing and sequence analysis

The DNA sequence of each clone was determined using a Big Dye terminator FS cycle sequencing kit. The sequence of the insert cDNA was determined using a 3100 Genetic Analyzer (Applied Biosystems Japan). Nucleotide sequences from each positive clone were compared to those entered in the GenBank database using the BLAST sequence search and comparison program.

2.7. GST pull-down assays

One μg of the pGBKT7-ZHX1 (1–873) plasmid was incubated at 30°C for 60 min with the T7 TNT Quick-coupled transcription/translation system in the presence of ^{35}S-methionine. The in vitro-synthesized product was then subjected to GST pull-down assays. TOPP3 cells were transformed with the pGEX-5X-1 or pGST-ZHX1 (1–873) fusion protein expression plasmid. The preparation of the GST-fusion protein and pull-down analysis have been described previously (Yamada et al., 1999b). Finally, the beads were resuspended in an equal volume of 2 × sodium dodecyl sulfate (SDS) sample buffer and each supernatant was loaded on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel, along with a prestained molecular weight marker. The gel was dried and exposed to a FUJIX imaging plate (Kanagawa, Japan). Interaction signals were detected with the FUJIX BAS-2000 image analyzing system. The relative purity and amount of each fusion protein were determined by gel-staining with Coomassie Brilliant Blue R-250.

2.8. Rapid amplification of the cDNA ends (RACE)

To obtain the 5′ end of the rat ZHX1 cDNA, we employed a 5′-RACE method using rat brain marathon cDNA and the Advantage 2 PCR kit. Three gene-specific primers, GSP1, 5′-TTTCT ACGGG TGTAA GGACG GGAG-3′, GSP2, 5′-CTGGA GACAC TCTCT GCTCT AGC-3′, and GSP3, 5′-CCTGG GTGGT ATTTC AGATT ATGC-3′, were used. The 5′-RACE procedure was carried out according to the manufacturer's protocol. Amplified DNA fragments were subcloned into the pGEM-T Easy vector and their nucleotide sequences were determined.

2.9. Preparation and analysis of RNA

Total RNA was prepared from normal ovary, uterus, and gonadotropin-primed rat immature ovaries using the TRIZOL reagent. Total RNA (10 μg/lane) was subjected to 1 % denaturating agarose gel electrophoresis and then transferred to a Biodyne membrane (ICN Biomedicals, Inc., Glen Cove, NY). For prehybridization and hybridization, the ExpressHyb hybridization solution was used. The filter and rat Multiple Tissue Northern blot were hybridized with a 1-kb α-^{32}P dCTP-labeled *Eco*RI fragment of the rat ZHX1 cDNA or a 1.4-kb α-^{32}P dCTP-labeled *Bam*HI fragment of the rat upstream stimulatory factor (USF) 2 which was labeled with the BcaBest DNA labeling kit (Yamada et al., 2001). Conditions for prehybridization, hybridization, and washing procedures were performed according to the protocol provided by the supplier. The blot was exposed to a FUJIX imaging plate. Hybridization signals were detected with the FUJIX BAS-2000 image analyzing system.

2.10. Cell culture and DNA transfections

Rat granulosa cells were isolated from DES-treated immature female rat ovaries and cultured as described previously (Hirakawa et al., 1999). Granulosa cells were plated into a 48-well dish and cultured for 24 h in hormone-free conditions.

HEK293 cells were cultured in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. A total of 5×10^4 cells per well were inoculated in a 24 well plate on the day prior to transfection.

DNA transfections were carried out using the FuGENE6 for granulosa cells or LIPOFECTAMINE PLUS for HEK293 cells. All plasmids used for the transfection were prepared using a QIAGEN plasmid kit, followed by CsCl gradient ultracentrifugation. The indicated amount of a GFP plasmid was used. In the case of HEK293 cells, 3 h after transfection, the medium was changed. The cells were observed with a fluorescence microscope (Olympus IX-70) (Inazu et al., 1999).

3. Results

3.1. Screening of ZHX1-interaction proteins

To analyze the molecular mechanism of the transcriptional role by ZHX1 in rat ovarian granulosa cells, we examined the interactions of human ZHX1 with either a known or a novel transcription factor. An entire coding sequence of the human ZHX1 was fused to the GAL4 DBD and this chimeric protein was employed as a bait to screen a rat

granulosa cell cDNA library using the yeast two-hybrid system. Approximately eleven million independent clones were screened, and 25 of these showed reproducible *HIS3*-, *ADE2*-, *MEL1*-positive properties, and β-galactosidase activity, respectively. We isolated plasmids that encode the GAL4 AD fusion protein from these clones. After a determination of their nucleotide sequences, they were compared with the GenBank database using the BLAST search program. Very interestingly, four clones, G1, G3, G8, and G92, were found to encode the rat homologue of ZHX1. A detailed nucleotide sequence analysis showed that G1, G3, and G8 were identical with one another. The DNA sequence of these clones contains an A residue stretch of 9 bp at the 3′-terminus of the cDNA that most likely corresponds to a portion of the poly(A)$^+$ tail of the ZHX1 messenger RNA (mRNA). In contrast, the 5′-terminus has a sequence that corresponds to the codon for the leucine at position 151 of mouse and human ZHX1. The nucleotide sequence of clone G92 encoded the amino acid sequence between 32 and 434 of mouse ZHX1. We focused on the analysis of these clones in this study. The other clones are currently undergoing further analysis and the results will be described in a future report.

We then examined the specificity of the dimerization of ZHX1 using the yeast two-hybrid assay (Table 1). We employed two bait plasmids, the pGBKT7 which expresses the GAL4 DBD alone, and the pGBKT7-ZHX1(1–873) which was used for screening, and three prey plasmids, pACT2 which expresses the GAL4 AD, and G8 and G92, which express the GAL4 AD fused to the cloned ZHX1 cDNAs. We determined β-galactosidase activity in yeast SFY526 strains that harbor various combinations of these plasmids. As shown in Table 1, when the yeast SFY526 strain containing pGBKT7 was transformed with the pACT2, G8, or G92, the transformants exhibited low but detectable β-galactosidase activities. Yeast harboring a combination of the pGBKT7-ZHX1 (1–873) and pACT2 also showed a low β-galactosidase activity. In contrast, yeast containing combinations of the pGBKT7-ZHX1 (1–873) and G8 or G92 expressed higher β-galactosidase activity than the other yeasts tested.

We next used in vitro GST binding assays to test for the dimerization of ZHX1. We employed two plasmids, the pGEX-5X-1, which express GST alone, and the pGST-ZHX1 (1–873), which express an entire coding sequence of ZHX1 protein fused to the GST. In vitro-translated, ^{35}S-labeled ZHX1 was incubated with purified GST or GST-ZHX1 (1–873) proteins bound to glutathione-sepharose. ZHX1 was found to bind GST-ZHX1 (1–873) but not to GST alone (Fig. 1). In contrast, an unprogrammed reticulocyte lysate failed to bind to either protein (data not shown). These results indicate that ZHX1 per se is able to form a homodimer both in vivo and in vitro.

Table 1
β-galactosidase activities of yeast strains harboring various combinations of GAL4 DBD- and AD-fusion plasmids

DBD-fusion plasmid	AD-fusion plasmid	β-galactosidase activity (U)
pGBKT7	pACT2	0.7 ± 0.1
pGBKT7	G8	3.4 ± 0.5
pGBKT7	G92	1.3 ± 0.1
pGBKT7-ZHX1 (1–873)	pACT2	0.7 ± 0.1
pGBKT7-ZHX1 (1–873)	G8	126.2 ± 9.9
pGBKT7-ZHX1 (1–873)	G92	12.6 ± 0.9

3.2. Comparison of amino acid sequence among rat, mouse, and human ZHX1

In order to isolate the 5′-non-coding sequence and a part of coding region of the rat ZHX1 cDNA that is missing in our obtained clones, we next employed a 5′-RACE method. Using combinations of gene-specific primers and adaptor primers, a cDNA fragment was obtained by PCR using rat brain marathon cDNA as a template. Finally, the size of the full-length rat ZHX1 cDNA was determined to be 4539 bp. A nucleotide sequence of the rat ZHX1 cDNA has been submitted to the DNA Data Bank of Japan Accession No. AB072439. The cDNA contains an open reading frame of 873 amino acids residues, which is shown in Fig. 2 in comparison with mouse and human ZHX1. The rat ZHX1 protein has a predicted molecular mass of 97.6 kilodaltons and an isoelectric point of 5.59. The rat ZHX1 sequence also contains motifs for two C-x_2-C-$(x)_{12}$-H-x_4-H Znf and five HDs (Fig. 2).

3.3. Tissue distribution of rat ZHX1 mRNA

The tissue distribution of rat ZHX1 mRNA was determined by Northern blot analysis (Fig. 3A). Approximately 4.7-kb ZHX1 transcript was observed in all rat tissues examined, although the intensity of the transcript varies among

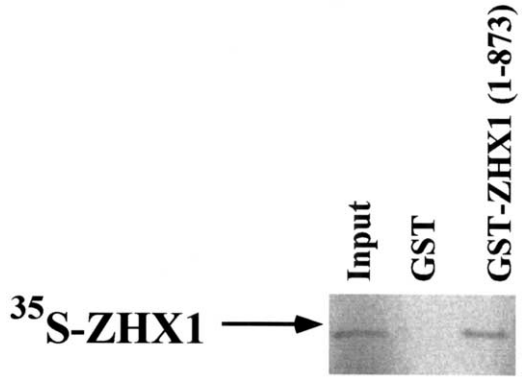

Fig. 1. GST pull-down assays. In vitro-translated, ^{35}S-labeled, ZHX1 was incubated with sepharose beads containing bound GST or the GST-ZHX1 (1–873) fusion protein. The beads were washed thoroughly and the bound protein was eluted and analyzed by SDS-PAGE. The gel was dried and exposed to a FUJIX imaging plate. Interaction signals were detected with the FUJIX BAS-2000 image analyzing system. The signal in the lane marked 'Input' represents 10% of the protein added to the reactions shown in the other lanes.

Fig. 2. Amino acid sequence of rat ZHX1 and a comparison with mouse and human ZHX1. The ZHX1 amino acid sequences of rat, mouse, and human are compared. The dashes indicate amino acid identity. The two zinc-finger motifs and five homeodomains are indicated by asterisks and double underlines, respectively. The acidic region and putative nuclear localization signals are marked with plus signs and closed circles (●), respectively.

these tissues. This result indicates that rat ZHX1 mRNA is expressed ubiquitously.

We then analyzed the regulation of expression of ZHX1 mRNA in the rat ovary (Fig. 3B). The administration of eCG to the immature female rat leads to the promotion of follicular development and differentiation. The issue of whether the level of ZHX1 mRNA is altered in eCG-primed immature rat ovary was also examined. As shown in Fig. 3B, the

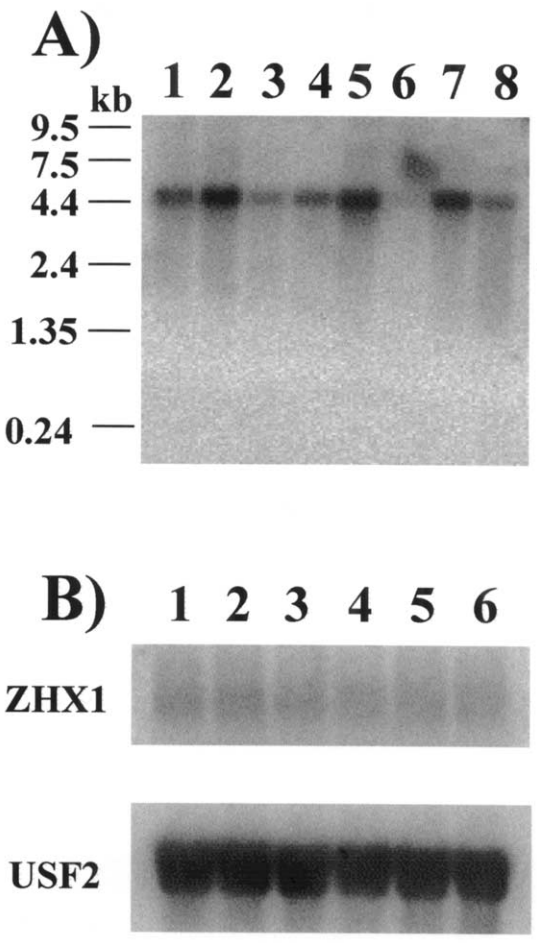

Fig. 3. Tissue distribution of rat ZHX1 mRNA and its regulation in ovary administrated with gonadotropins. (A) A rat multiple tissue northern blot (Clontech) was hybridized with ^{32}P-labeled rat ZHX1 cDNA. Each lane contains 2 μg of poly(A)$^+$-RNA isolated from indicated tissues. Size markers are shown on the *left* in kb. Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. (B) Ten μg of total RNAs isolated from uterus, normal ovary and eCG- alone or eCG- then followed by hCG-primed rat ovaries was subjected to 1% denaturating agarose gel electrophoresis. Blotting and hybridization procedures are described in Section 2 with ^{32}P-labeled ZHX1 (*Upper*) or USF2 probe (*Lower*). Lane 1, uterus; lane 2, normal ovary; lanes 3 and 4, ovary administrated with eCG for 24 and 48 h, respectively; lanes 5 and 6, ovary administrated with eCG for 48 h, followed by administrated with hCG for 24 and 48 h, respectively.

level of ZHX1 mRNA was not altered within 48 h after eCG treatment. When hCG was injected at 48 h after administration of eCG, both ovulation and luteinization were initiated in the rat ovary. In addition, the level of ZHX1 mRNA was not altered in this treatment. In these treatments, the level of USF2 mRNA as an internal control was also not changed. These results indicate that the level of ZHX1 mRNA is not regulated by gonadotropins.

3.4. Subcellular localization of ZHX1

To determine the subcellular localization of the ZHX1 protein, we prepared a GFP-ZHX1 fusion protein expression plasmid. When the pEGFP-C1E1 plasmid encoding GFP alone was transfected into HEK293 cells, the corresponding protein was observed in the cytoplasm (Figs. 4A,B). In contrast, when the pGFP-ZHX1 (1–873) plasmid in which an entire coding sequence of ZHX1 was fused to the C-terminal of GFP, was transfected, the fusion protein was observed in the nuclei (Figs. 4C,D). These results indicate that ZHX1 is also able to localize in the nuclei as a GFP fusion protein.

4. Discussion

The rat ZHX1 cDNA was cloned as one of ZHX1-interacting proteins from a granulosa cell cDNA library using the yeast two-hybrid system. The homology of the amino acid sequence of rat ZHX1 with human or mouse ZHX1 protein was 92.6 and 96.7%, respectively. The two Znf- and five HD-motifs are conserved among these species. While the amino acid sequence of the Znf-motifs and HD1 and 2 were identical among these species, that of HD3–5, were slightly different from each other. The Znf motif is a well-characterized one that is required for binding to the cognate DNA sequence via a Zn^{2+} molecule (Philipsen and Suske, 1999). HD contains a conserved 60 amino acid motif (Gehring et al., 1994). Although HD was originally reported as DBD, it is known to be an interaction domain with other transcription factors or molecules involved in the signal transduction pathway (Kim et al., 1998; Perrone et al., 1999; Hiroi et al., 2001). In the case of the human ZHX1, a region containing HD1 through HD2 indeed interacts with a glutamine-rich region of NF-YA (Yamada et al., 1999a).

A number of transcription factors such as members of the CCAAT/Enhancer-binding protein family, nuclear receptors, and E box (5′-CACGTG-3′)-binding proteins, interact with each other and form homo- or hetero-dimers (Vinson et al., 1993; Massari and Murre, 2000; Aranda and Pascual, 2001). Our results from the yeast two-hybrid assays and GST pull-down assays clearly show that ZHX1 forms a homodimer both in vivo and in vitro. Thus, we conclude that ZHX1 not only associates with NF-YA but also forms a homodimer. Dimerization of transcription factors occurs via certain motifs including leucine zipper, helix-loop-helix, or etc. The G92 encoded the amino acid sequence from 32 to 434 that contains the Znf1 to HD1 of rat ZHX1. It indicates that the Znf1 to HD1 is required for dimerization.

The cloned insert was 4539 bp in size, nearly the same as the transcript of ZHX1, as determined by Northern blot analysis. The level of ZHX1 mRNA is highly expressed in heart, brain, liver, kidney, testis, and in lower amounts in spleen, lung and skeletal muscle. Expression is also observed in the rat uterus and ovary. Accordingly, rat ZHX1 mRNA is expressed ubiquitously as well as human and mouse ZHX1 mRNAs. The level of ZHX1 mRNA was not altered in gonadotropin-administrated immature rat ovaries.

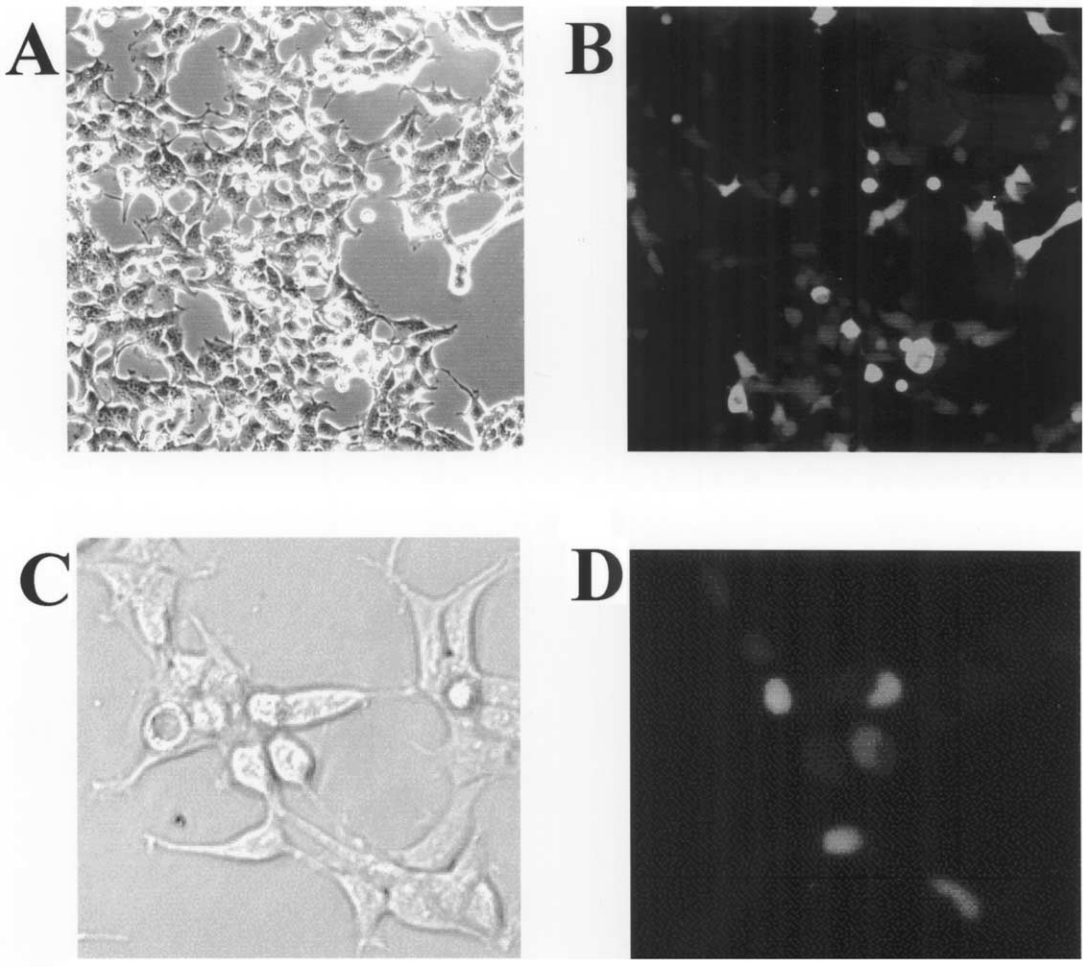

Fig. 4. Nuclear localization of ZHX1. Expression plasmids (300 ng) which encode GFP alone (A and B); or full-length ZHX1 proteins fused to the C-terminal of GFP (C and D) were transfected into HEK293 cells. Forty eight h after transfection, subcellular localization of GFP fusion protein were observed. Panels A and C, phase contrast microscopy photographs. Panels B and D, fluorescence microscopy photographs of the same fields as A and C, respectively.

An extensive analysis of the amino acid sequence revealed the presence of three putative nuclear localization signals (NLSs) and an acidic region in the C-terminal region. When GFP-ZHX1 but not GFP was expressed in granulosa cells, green fluorescence signals were observed the cells which were detached from the dish (data not shown). We conclude that the overexpression of GFP-ZHX1 was toxic to granulosa cells since the overexpression of exogenous protein in these cells sometimes led to cell death for unknown reasons. As a result, we selected HEK293 cells as an alternate system. It has been shown that the GFP-ZHX1 fusion protein is localized in the nuclei of HEK293 cells. Three putative NLSs exist in the amino acid sequences 469–473, 741–765, and 861–867. The issue of whether either or all the sequence is required for nuclear localization remains to be determined. The acidic region of most transcription factors has transcription stimulation activity (White et al., 1991). It has been reported that transcription regulatory regions interact with co-factors to function as transcriptional activators or repressors (Fry and Farnham, 1999; Hu and Lazar, 2000). Two classes of co-factors are known; one is a co-activator(s) such as the p300/cyclic AMP response element-binding protein-binding protein that exhibits histone acetylase activity per se, and the other is a co-repressor(s) such as the nuclear co-repressor, mSin3, and histone deacetylases (Hu and Lazar, 2000; Wolffe et al., 2000; Aranda and Pascual, 2001). Although these co-factors are expressed ubiquitously, they play an important transcriptional role in a gene-specific manner via their interactions with cell type-specific or ligand-dependent transcription factors (Fry and Farnham, 1999; Hu and Lazar, 2000). ZHX1 is also ubiquitously expressed, and, thus, could be involved in the formation of highly organized nucleoprotein complexes that participate in the regulation of many genes in various tissues as well as granulosa cells. This is the case where ZHX1 might be part of a transcription factor network.

In summary, we report on the cloning and characterization of rat ZHX1 as an interacting protein of ZHX1. The issue of whether dimerization of ZHX1 interfere with or enhance an association of ZHX1 with NF-YA, whether ZHX1 is a transcriptional activator or repressor, and

which gene expression is regulated by ZHX1 remains to be determined. Further studies will be required to address these questions.

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