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Molecular Cloning of a Novel 130-kDa Cytoplasmic Protein, Ankhzn, Containing Ankyrin Repeats Hooked to a Zinc Finger Motif[1]

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A novel gene was trapped in mouse embryonic stem cells with a promoterless gene trap vector. Fused transcripts were isolated from the embryos by rapid amplification of cDNA ends, which were used for full-length cDNA cloning. The protein predicted from the cDNA consisting of 7143 nucleotides comprises 1184 amino acids, which was confirmed by *in vitro* transcription/translation assaying. An antibody against the synthesized peptide reacted with an approximate 130-kDa protein on SDS-PAGE. A search of available databases revealed that this protein is a novel protein composed of 17 ankyrin repeats hooked to a zinc finger motif, which we named Ankhzn. Ankhzn was observed on the endosomal membrane on immunoelectron microscopic analysis. Ankhzn belongs to a new subgroup of double zinc finger proteins which may be involved in vesicle or protein transport. Ankhzn mRNA and its protein were expressed ubiquitously from embryonic day 10.5 to adulthood.

Much biological interest has been focused on tissue-specific and stage-specific gene expression, and their regulation *in vitro* or *in vivo*. The gene trap (GT) method involving mouse embryonic stem (ES) cells provides a combined strategy for the simultaneous identification of a gene integrated into the mouse genome and characterization of the phenotype of mutant mice (1–3). Gene trapping is also a useful method for screening a novel gene expressed in a spatiotemporal-specific manner during development and differentiation. At the same time, gene trapping provides an approach for studying the ubiquitous expression of a novel gene involved in basic cellular functions (4) according to random insertion of a promoterless GT vector into the genome of ES cells, in which many genes are expressed for cell growth and proliferation.

We introduced the GT vector into ES cells, a GT 3-12 ES cell line was established (5) and ubiquitous lacZ expression was observed in the GT 3-12 heterozygous mice. The fusion transcript was isolated from embryonic day 16.5 (E16.5) on 5′ rapid amplification of cDNA ends (5′RACE). The 5′ end comprising 31 nucleotides was used for full length cDNA cloning by means of primer walking. A search of available databases revealed that the cloned cDNA is novel and there is no homologue in other species. The protein predicted from the cloned cDNA comprises 1184 amino acids with an apparent molecular mass of 130 kDa, and has neither a hydrophobic signal sequence nor a transmembrane domain. The 130 kDa-protein shows unique structural features, *i.e.* there are 17 ankyrin repeats in the middle, and a double zinc finger motif in its C-terminal part. This unique structure has not previously been reported. We named this protein Ankhzn (*ank*yrin repeats *h*ooked to a *zinc* finger motif). We report here the cDNA cloning, molecular characterization and intracellular localization of a novel 130-kDa cytoplasmic protein, Ankhzn.

MATERIALS AND METHODS

5′ RACE and cDNA cloning. Fusion transcripts with the promoterless GT vector (5), SA-*neo*-IRES-*NLSlacZ*, were identified by 5′ RACE (6) using total RNA from GT 3-12 mouse brains at E16.5. The first strand cDNA was synthesized with the *neo*-specific primer, neo2 (5′-GTCCAGATCATCCTGATCGACA-3′), and dATPs were added at the 3′ end of the first strand cDNAs with terminal deoxynucleotidyl transferase. The dA-tailed cDNA was subjected to two rounds of nested PCR amplification. For the first round, using 10 pmol each of neo2 primer and oligo(dT)18 adapter primer, XT-18 (5′-CCCTCG-AGGTCGACGGTATCGTTTTTTTTTTTTTTTTT-3′), one cycle of PCR (94°C for 1 min, 42°C for 2 min, 72°C for 5 min) and then 2 more cycles (94°C for 1 min, 48°C for 2 min, 72°C for 5 min) were carried

[1] The nucleotide sequence(s) reported in this paper has been deposited with the GenBank database under Accession No. AB011370.

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out in a total volume of 50 μl. After the 3 cycles of PCR, 15 pmol of neo2 primer and 50 pmol of KS primer (Stratagene), 5′-TCGAGGTCGACGGTATC-3′, were added, followed by 30 cycles of PCR (94°C for 1 min, 65°C for 2 min, 72°C for 5 min) in a final volume of 100 μl. For the second round PCR, an aliquot of the first-round PCR products was added to a solution containing 50 pmol of the gene-specific primer, neo1 (5′-CGAGATCTGTTGTGCCCAGTCATAG-3′), and 50 pmol of KS primer under the conditions for the first-round PCR. The PCR products were digested with *Bgl*II and *Sal*I, subcloned into the pBluescript II KS(−) vector, and then sequenced.

The 31 bp nucleotide sequence obtained on 5′ RACE was used for full length cDNA cloning. The first strand cDNAs were synthesized with an adapter primer containing a random decamer (5′-AAGAATTCGCGGCCGCAGGAAN10-3′), and a cDNA library was prepared with a marathon cDNA amplification kit (Clontech). We designed gene specific primers GT 3-12-1 (1-20), GT 3-12-3 (1158-1177), GT 3-12-4 (2772-2791), GT 3-12-9 (3941-3959), GT 3-12-17 (5453-5473) and GT 3-12-16 (complementary, 6947-6978), these nucleotide positions being those in the Ankhzn cDNA sequence deposited in GenBank under accession No. AB011370, about 100 bp upstream from the 3′ end of each cloned fragment. This overlapping region was checked to determine whether the cloned plasmid had the correct sequence.

Transcription/translation. The FLAG tagged-plasmid, pCFLAG, was constructed using synthesized oligonucleotides corresponding to the FLAG octapeptide, DYKDDDDK, followed by stop codons, a *Sal*I site and a *BamH*I adaptor, (sense, 5′ GGGAGACTACAAGGACGACGACGACAAGTGATTGATTGAGTCGACG-3′; antisense, 5′-GATCCGTCGACTCAATCAATCACTTGTCGTCGTCCTTGTAGTCTCCC-3′). These oligonuleotides were annealed and ligated into the *Sma*I and *BamH*I sites of pBluescript II SK(−). The N-terminal Ankhzn cDNA was obtained by PCR with a primer set, GT 3-12-1 (5′-GGAGGAAGGGCCGGGCCCGA-3′) and GT 3-12-6 (5′-ACAGTCGTTCCTCCGCATG-3′), and the cloned cDNA as a template. The resulting PCR product was subcloned, sequenced, verified to be correct and then an *Eco*RI-fragment was inserted into the *Eco*RI site between the T7 promoter and the FLAG-epitope sequence of pCFLAG. The FLAG-tagged N-terminal Ankhzn cDNA and its reverse orientation were designated as pAz(1/6)CFLAG and pAz(6/1)CFLAG, respectively. These plasmids were incubated with T7 RNA polymerase, an amino acid mixture, [^{35}S]-methionine (Amersham), RNasin and TNT rabbit reticulocyte lysate (Promega). Samples were subjected to SDS-PAGE, dried and then visualized by autoradiography.

DNA transfection. pCAG-Az(1/6)CFLAG was constructed by insertion of the *Sal*I-fragment of pAz(1/6)CFLAG into the unique *Xho*I site of a eukaryotic expression vector, pCAGGS (7). B103 cells, provided by Dr. D. Schubert (8), were co-transfected with pCAG-Az(1/6)CFLAG and Moloney murine leukemia virus promoter drived *NLS-lacZ*. Cells were fixed in 4% paraformaldehyde in PBS, stained with a X-gal staining solution overnight and then immunostained with mouse monoclonal antibodies against the FLAG peptide (Eastman Kodak Co.) and HRP-labeled swine anti-mouse immunoglobulin G (Dako).

Western blot analyses. The peptide sequence in frame (GT 3-12-b, KKEGEEEARDGQTPLH) was designed from EST (GenBank Accession No. AA111267), synthesized, conjugated with keyhole limpet hemocyanin (Calbiochem-Novabichem), and then injected subcutaneously into rabbits. Antiserum was purified by affinity chromatography on a peptide-coupled Sepharose-4B column. Several tissues and cultured cells were homogenized in 20 mM Tris-HCl (pH 7.5) buffer containing 0.1 mM phenylmethylsulfonyl fluoride, followed by centrifugation for 10 min at 4,000 rpm. Soluble proteins and membrane fractions were prepared from the low speed supernatant by centrifugation at 100,000 × g for 60 min. The protein concentrations were determined by means of the Bradford protein assay (Bio-Rad), and 50 μg protein samples were fractionated by 7.5% SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked with 5% skim milk in PBS, and then incubated overnight with the purified anti-Ankhzn-b antibodies (1:200 dilution) at 4°C. Each blot was washed with 0.1% Tween-20 in PBS, followed by reaction with HRP-labeled swine anti-rabbit immunoglobulin G (Dako) for 30 min and then developed with an ECL detection system (Amersham).

Northern blot analyses. Total RNA was isolated with ISOGEN (Wako Chemical, Japan), fractionated on a formaldehyde agarose gel, and then hybridized with a [^{32}P]-labeled cDNA probe of the 3′ non-translated region (nucleotide positions 3942-7143) at 42°C in a 40% formamide solution.

Immunocytochemical analyses. B103 cells were grown on plastic dishes, fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 for 30 min at room temperature. The cells were incubated with rabbit anti-Ankhzn-b antibodies, biotin-labeled goat anti-rabbit immunoglobulin and HRP-conjugated streptavidine (Amersham), followed by reaction with 3-3′ diaminobenzine tetrahydrochloride and H_2O_2. After treatment with osmium tetroxide, the cells were embedded in Epon and subjected to immuno-electron microscopic analysis.

RESULTS

Cloning of the full-length cDNA. In order to identify the trapped gene we performed cDNA cloning, by means of 5′ RACE, of fusion transcripts from E16.5 brains. The longest extended cDNA to the 5′ end contained a 31 bp nucleotide, 5′-GGAGGAAGGGCCGGGCCCGACATGGCGGAAG-3′, which was followed by the GT vector, indicating that the 31 bp sequence is the first exon. Gene-specific primers, GT 3-12-1 and its nested primer, GT 3-12-2 (5′-CGGGGCCCGACATGGCGGAAG-3′), were designed in the 31 bp for full length cDNA cloning. The overlapping cDNAs were amplified by PCR, through the GT 3-12 gene-specific primers and a random decamer with an anchor primer, subcloned and then sequenced. Consequently, the full-length cDNA obtained comprised 7160 bp, and carried a poly A sequence and its 22 bp upstream polyadenylation consensus sequence, AATAAA. (GenBank accession No. AB011370).

The predicted amino acid sequence. The first ATG codon at position 22 of the Ankhzn cDNA was assigned as the initiator methionine, which conforms favorably to the Kozak consensus sequence for eukaryotic translation initiation (9). The open reading frame (ORF) was confirmed in two ways; coupled *in vitro* transcription/translation and FLAG-epitope expression in cultured cells to determine the initial methionine and reading frame, respectively (Fig. 1). Figure 1B shows a major band corresponding to a molecular mass 45 kDa, which matched the estimated size of the translation product of 397 amino acids from pCAG-Az(1/6)CFLAG (Fig. 1A) consisting of the N-terminus of the Ankhzn (1 to 381 amino acids, Fig. 2) and the FLAG-peptide, indicating that the first ATG at nucleotide position 22 acts as a translation initiation. The FLAG-epitope in frame was expressed in cultured B103 cells (Fig. 1C) co-transfected with pCAG-Az(1/6)CFLAG and the *lacZ* expression vector, while cells transfected with out of

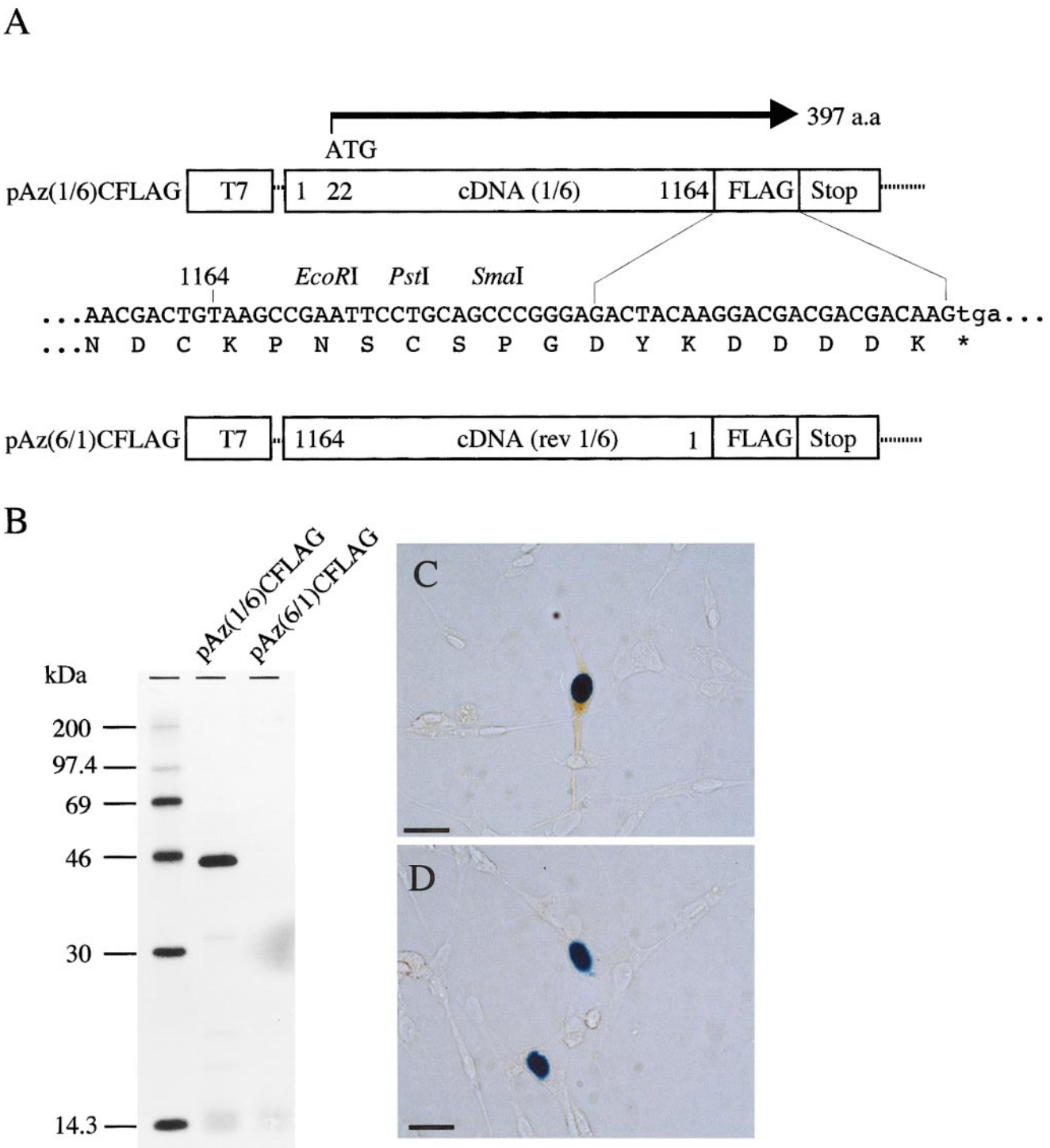

FIG. 1. (A and B) Coupled *in vitro* transcription/translation. Constructs of the FLAG-tagged N-terminal of Ankhzn (A). Translation products were analyzed by SDS-PAGE. Labeled molecular weight markers were run in parallel (B). (C and D) A plasmid carrying *NLS-lacZ* with the Moloney murine leukemia virus promoter was co-transfected with pCAG-Az(1/6)CFLAG. FLAG-epitope expression in frame (C), but not out of frame (D) was detected by immunostaining with mouse monoclonal anti-FLAG antibodies. Bars represent 20 μm in C and D.

frame FLAG-epitope were only expressed lacZ in these nuclei (Fig. 1D).

Further identification of the ORF was performed by Western blotting with antibodies against the synthesized peptide, GT 3-12-b, corresponding to amino acids 777 to 790 in the sequence shown in Fig. 2. A protein band corresponding to an apparent molecular mass of 130 kDa reacted with the anti-Ankhzn-b antibodies

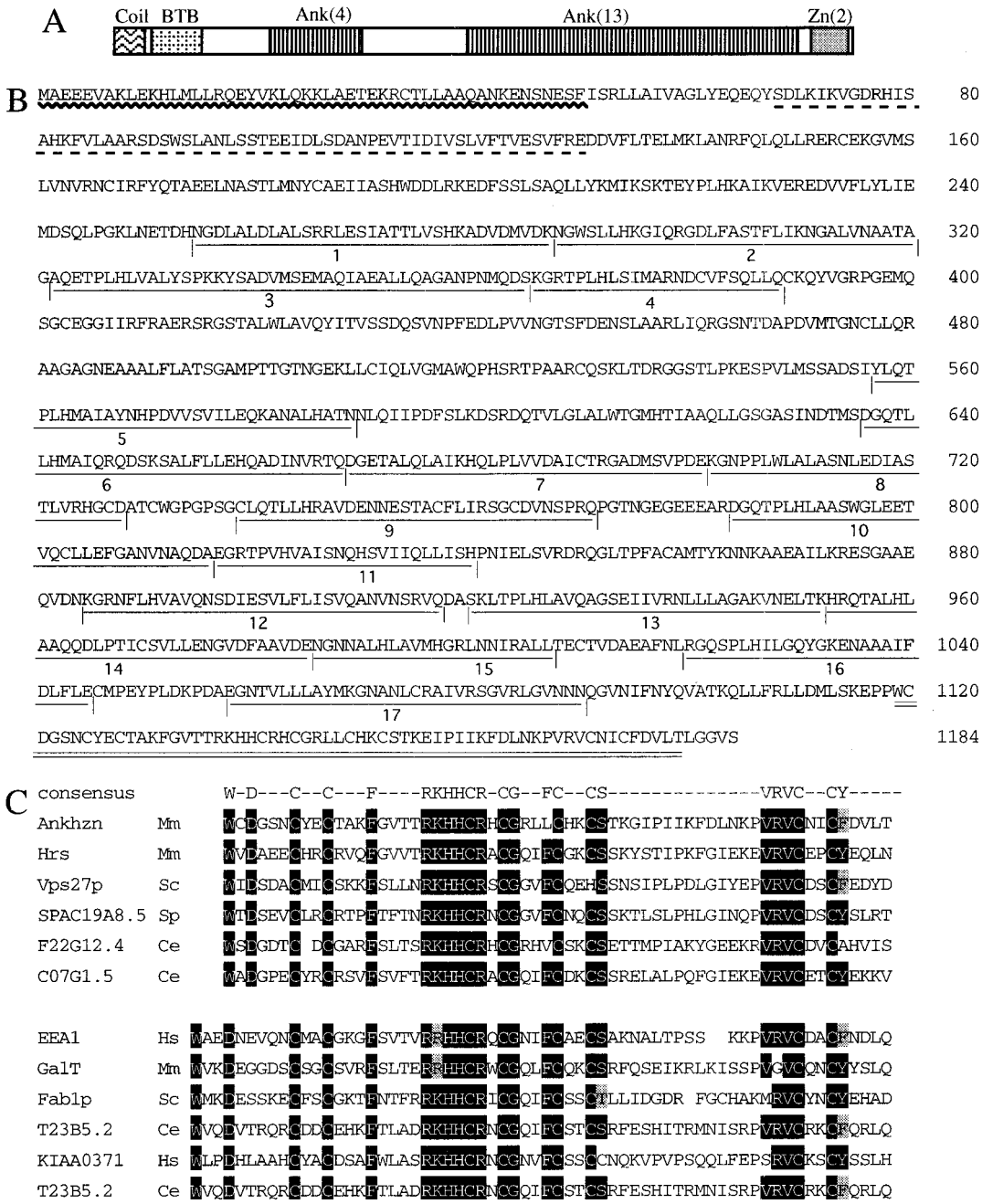

FIG. 2. (A) Domain structure of Ankhzn. The following domains are shown; Coil, coiled-coil domain; BTB, BTB/POZ domain; Ank (4) and Ank (13), ankyrin 4 and 13 repeats, respectively; Zn (2), double zinc finger domain. (B) Sequence of Ankhzn predicted from its cDNA. The wavy line, dashed line, underline, and double underline show coiled-coil motif, BTB/POZ domain, the 17 ankyrin repeats with their numbers (1 to 17), and the zinc finger motif, respectively. (C) Alignment of sequences related to the double zinc finger domain. Black and shaded backgrounds represent identical and conserved functional similar amino acids, respectively. Species abbreviations: Mm, mouse; Sc, *Saccharomyces cerevisiae;* Sp, *Schizosaccharomyces pombe;* Ce, *C. elegans;* Hs, human.

(Fig. 3), which is consistent with the calculated average molecular weight of 1,297,989 predicted from the cDNA. These results indicate that the Ankhzn cDNA contains a 21 bp 5′-untranslated region followed by an ORF of 3552 bp, which encodes 1184 amino acids, and a large 3′-untranslated region.

A comparison of the predicted protein sequence of Ankhzn with available databases revealed that Ankhzn is a novel protein with several characteristic features, a coiled-coil region (10), a BTB/POZ (bric-a-brac, tramtrack and broad complex/ poxvirus and zinc finger)-like domain (11, 12) at its N-terminal, ankyrin

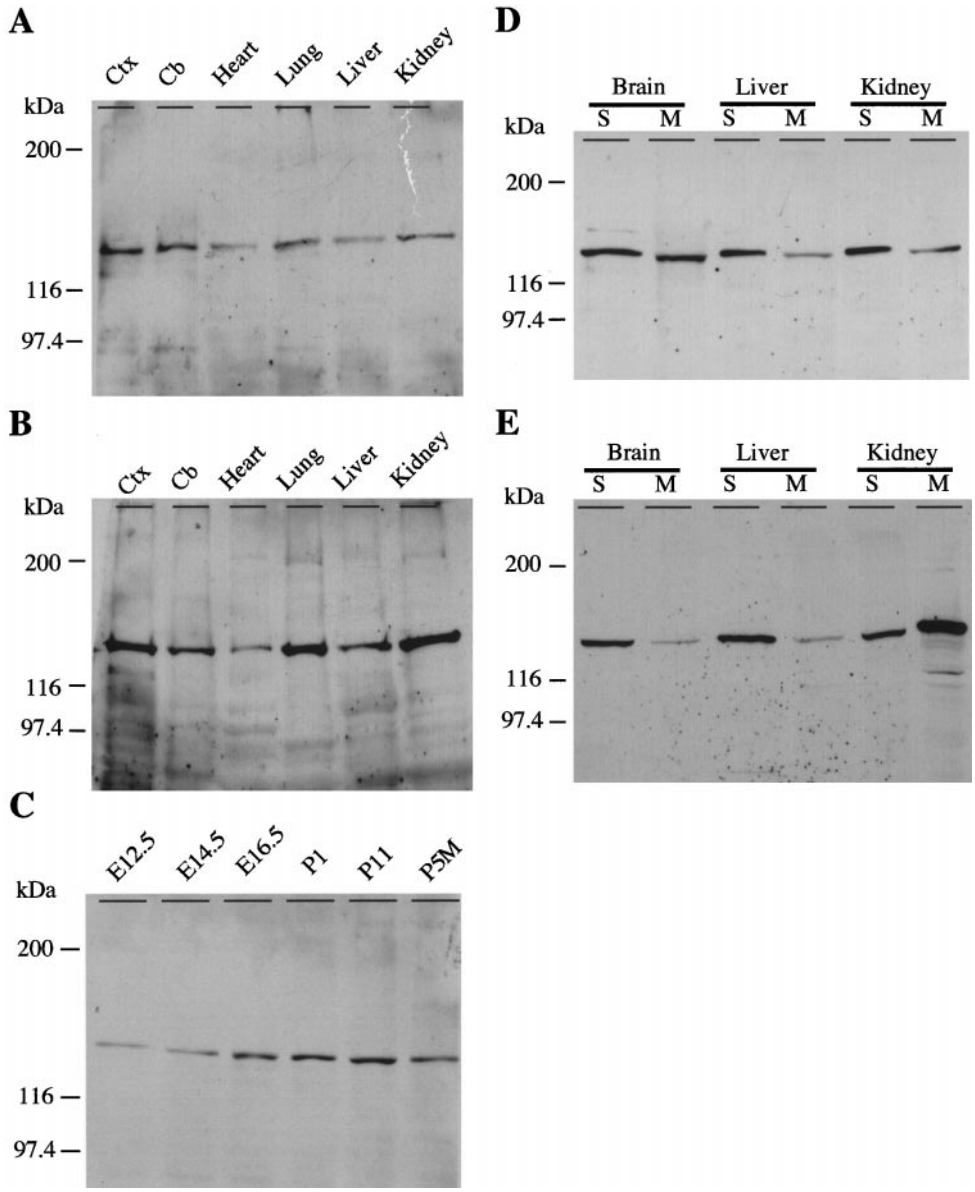

FIG. 3. Western blotting analyses of cerebral cortex (Ctx), cerebellum (Cb), heart, lung, liver, and kidney from newborn (A) and adult mice (B). (C) Developmental changes of Ankhzn in the brain from E12.5 to 5 months. (D and E) The soluble (S) and membrane fractions (M) from newborn (D) and adult (E) were subjected to Western blot analysis. The molecular mass markers are indicated on the left of the gels.

repeats in the middle, and a zinc finger motif in the C-terminal region (Fig. 2). Protein profile was examined by 'Pfscan' program of ISREC (Swiss Institute for Experimental Cancer Research). Ankhzn contains 17 divergent ankyrin-like repeats separated into two parts, 4 and 13 repeats, respectively. It aslo contains a double zinc finger motif which was found in proteins in yeast to man; mouse Hrs (13) (51% identity), yeast Vps27p (14) (43% identity), human EEA1 (15) (40% identity), β 1-4 galactosyltransferase (GalT) (16) (40% identity), and Fab1p (17) (31% identity). The zinc finger domain is composed of 8 cysteines, four amino acid residues between the zinc fingers, a well conserved basic amino acid sequence, such as R/KHHCR, and characteristic amino acids, tryptophan, phenylalanine and histidine (Fig. 2C).

Expression of Ankhzn. The expression pattern as to the protein level was examined by Western blot analysis using anti-Ankhzn-b antibodies (Fig. 3). An immuno-reactive band corresponding to a molecular mass of 130 kDa was detected for all tissues examined and developmental stages of the brain. Ankhzn associated with membranes increases in adult kidney and decreases in adult brain Ankhzn occurs in both the soluble and membrane fractions, indicating that a part

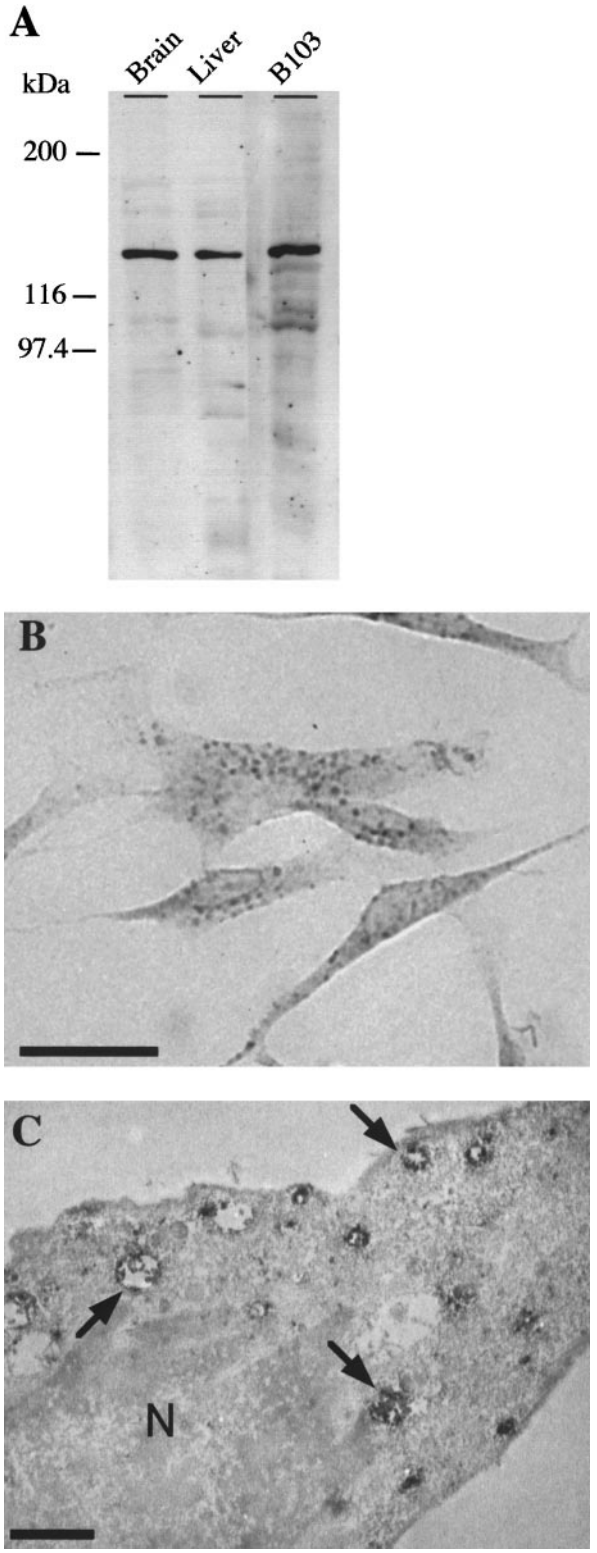

FIG. 4. Intracellualar localization of Ankhzn. (A) Low speed supernatants from mouse brain, liver, and B103 cells were separated by SDS-PAGE. Molecular mass standards are indicated on the left. (B) B103 cells were fixed, permeabilized, incubated with anti-GT 3-12-b antibodies, and then processed for immunoperoxidase microscopy using a biotin-streptavidin reaction system. (C) After peroxidase reaction, an ultrathin section was subjected to immunoelectron microscopic analysis. Arrows indicate endosomes. N; nucleus. Bars represent 20 μm in B and 2 μm in C.

of Ankhzn is associated with the membranes without a transmembrane domain.

As a first step toward functional characterization of Ankhzn, we examined its intracellular localization in several cell lines. B103 cells, which endogenously express Ankhzn (Fig. 4A), were subjected to immunocytochemistry. Immunostaining with anti-Ankhzn-b antibodies after the cells were permeabilized with Triton X-100 demonstrated that Ankhzn is associated with intracellular vesicles throughout the cells and soluble forms of Ankhzn are seemed to leak partially during permeabilization of the cells (Fig. 4B). Immunoelectron microscopy showed that Ankhzns surround endosomes, not the nuclear or plasma membranes. Some are on the small vesicles like coated vesicles in the early endosomes close to the plasma membrane in the endocytotic pathway (Fig. 4C).

To analyze Ankhzn transcripts, total RNAs from several tissues of wild type mice were hybridized with the 3′ untranslated region. A single transcript of about 7 kb was detected. The expression of Ankhzn mRNA was widely distributed in mouse tissues. To determine whether or not *Ankhzn* expression is regulated developmentally, we examined the levels of total RNA isolated from newborn tissues (Fig. 5A) and of postnatal 21 days (Fig. 5B), and the brain at E10.5, E12.5, E14.5, E16.5, birth, P7, P14, P21, P40 and 5 months (5M) (Fig. 5C). At all stages examined, widespread expression of the *Ankhzn* gene was observed.

DISCUSSION

Here we describe molecular characterizations of a new gene, *Ankhzn*, identified by gene trapping in ES cells. The GT vector was inserted within an active transcription unit and expressed as a fusion transcript. A 31 nucleotide sequence was obtained from the fusion transcript by 5′ RACE and used as the primer design for cDNA cloning. Sequence analysis of the full-length cDNA, and its comparison with available gene and protein databases revealed that the trapped gene is a novel one. The amino acid sequence of Ankhzn was found to show 29% identity to the 1107 amino acids predicted from the *F22G12.4* gene of *C. elegans* throughout the sequence (18) (GenBank accession No. Z92831), and the function of the putative protein derived from the *F22G12.4* gene is not yet known. Some mouse ESTs (GenBank Accession Nos. W65001, AA111267, W78522, W21027, AA474749, AA260903, AA501074 and R94364) exhibit high homology with a part of Ankhzn (97.9-99.4%). The human EST clone

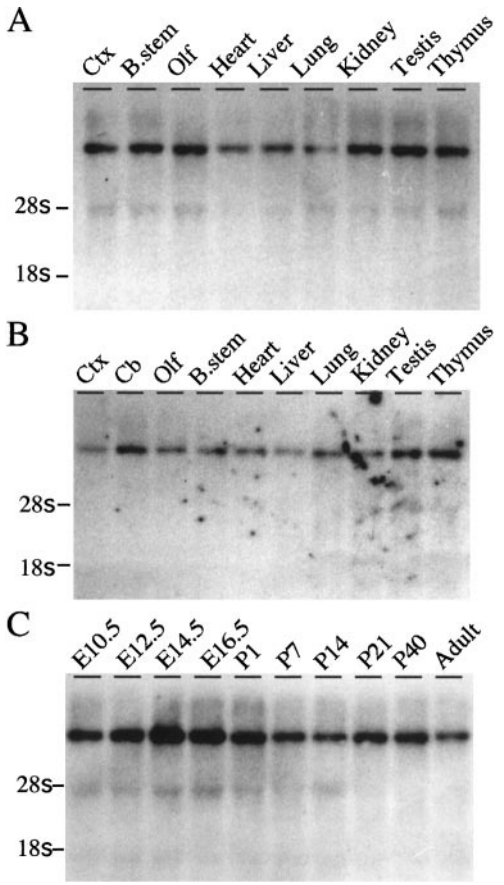

FIG. 5. Northern blot analyses. The tissue source of the mRNA (20 μg/lane) in each lane is indicated at the top; cerebral cortex (Ctx), cerebellum (Cb), olfactory bulb (Olf), brain stem (B. stem), heart, liver, lung, kidney, testis, and thymus from newborn (A) and P 21 mice (B). (C) Developmental changes of mRNA in the brain from E10.5 to adulthood.

(GenBank accession No. AA399607) is highly homologous to the coding region of Ankhzn (nucleotide positions 3080-3373, 84.5%), which means that *Ankhzn* is conserved between mouse and man. Ankhzn or a related protein such as the putative gene product of *F22G12.4* is thought to be widely distributed from *C. elegans* to man, although its protein or cDNA has not cloned from other species.

The predicted 1184 amino acid sequence exhibits the structural feature of 17 ankyrin repeats hooked to a zinc finger motif. Ankyrin repeats are present in a large number of functionally diverse membrane-associated proteins that mediate the linkage of the cytoskeleton and have been shown to be important for protein-protein interactions. The most highly conserved region in known proteins is a region of approximately 60 residues at the C-terminus, which may form a zinc binding site around two pairs of cysteine residues, CX2CX12CX2C and CX2CX18CX2C (Fig. 2C), different from in the cases of LIM domain (19) and RING finger (20). The double zinc-finger domain composed of eight cysteine residues in Ankhzn, WX5-8CX2CX3FX4R/R/KHHCRXCGX2FCX-2CSX12-14VRV CX2CF/Y, is well conserved among a variety of intracellular proteins; Vps27p (14), Hrs (13), Vac1p(21), Fab1p (17), GalT (16), and EEA1 (15, 22). Some of these zinc finger domains are involved in protein trafficking to the vacuoles of yeast like Vps27p, and to the endosomes in mammalian cells like EEA1. Vps27p controls membrane traffic through the prevacuole/endosomal compartment in yeast. Hrs associates with SNAP-25 and is implicated in regulation of the secretory process through the vesicle-trafficking protein complex (23). Ankhzn was also shown to be localized at least to the endosomes by immuno-electron microscopy. These findings suggest Ankhzn may be involved in intracellular protein/vesicle traffic through the endocytotic pathway.

In addition to the ankyrin repeats and double zinc finger domain, Ankhzn contains a coiled-coil motif at the N-terminus and a BTB/POZ-like domain. In the coiled-coil structure two helices bind each other to form a homodimer. Hrs, Vps27p, EEA1, Fab1p and Vac1p, which have the double zinc finger domains, also contain the coiled-coil regions in their middle. The structural significance of these proteins is not yet known. The BTB/POZ domain of Ankhzn consists of about 60 amino acids with 19% to 26% homology to proteins described below. The BTB/POZ domain is found primarily at the N-terminus of zinc finger proteins (11, 12), such as *Drosophila* kelch (24), ttk (25), *C. elegans* MEL-26 (26), vaccinia virus A55R (27), and human SPOP (28). The BTB domain mediates the protein dimerization *in vitro* of some transcription factors (12, 29), and was shown to be sufficient for the dimerization of *Drosophila* kelch (24) and PLZF oncoprotein (30). Furthermore, Ankhzn was observed in both the soluble and membrane fractions, like Hrs (31) and EEA1 (15). Judging from these findings, Ankhzn in part may function as a homodimer, albeit the Ankhzn dimer form remains to be shown, or as a protein complex that is reversibly associated mainly with membrane protein(s) on the endosomes. A number of studies have been focused on the vesicle or protein traffic in cells, however, a protein with stuctural features such as Ankhzn has not previously been reported. Further experiments are necessary to elucidate the molecular mechanism whereby Ankhzn is involved in endocytosis, and the recycling of vesicles and membrane proteins.

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