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Molecular Cloning and Characterization of a RING-H2 Finger Protein, ANAPC11, the Human Homolog of Yeast Apc11p

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Abstract Yeast Apc11p together with Rbx1 and Roc2/SAG define a new class of RING-H2 fingers in a superfamily of E3 ubiquitin ligases. The human homolog of Apc11p, ANAPC11 was identified during a large-scale partial sequencing of a human liver cancer cDNA library and partial characterization was performed. This 514 bp full-length cDNA has a predicted open reading frame (ORF) encoding 84 amino acids. The ORF codes for ANAPC11, the human anaphase promoting complex subunit 11 (yeast APC11 homolog), which possesses a RING-H2 finger motif and exhibits sequence similarity to subunits of E3 ubiquitin ligase complexes. In Northern blot hybridization with poly(A) RNA of various human tissues using radio-labelled ANAPC11 cDNA probe, we found strong signals detected in skeletal muscle and heart; moderate signals detected in brain, kidney, and liver; and detectable but low signals in colon, thymus, spleen, small intestine, placenta, lung, and peripheral blood leukocyte. The ANAPC11 gene is located at the human chromosome 17q25. ANAPC11 is distributed diffusely in the cytoplasm and nucleus with discrete accumulation in granular structures in all the cell lines (AML 12, HepG2, and C2C12) transfected. Expression level of ANAPC11 is found higher in certain types of cancer determined in the RNA dot blot experiment. J. Cell. Biochem. 83: 249–258, 2001.

Key words: RING-H2 finger motif; ANAPC11; anaphase promoting complex; liver cDNA

RING finger motif was first identified as a new class of zinc fingers a decade ago in the protein product of the human gene Really Interesting New Gene 1 (RING1) [Freemont et al., 1991]. They share a consensus sequence of Cys-X_2-Cys-X_{9-39}-Cys-X_{1-3}-His-X_{2-3}-Cys/His-X_2-Cys-X_{4-48}-Cys-X_2-Cys where X can be any amino acid and in which Cys and His represent the zinc binding residues in a unique "cross-brace" arrangement. RING finger proteins are subcategorized into RING-(HC) and RING-H2, depending on whether a Cys or His occupies the fifth co-ordination site [Lovering et al., 1993; Borden and Freemont, 1996; Saurin et al., 1996]. These proteins are found in a plethora of eukaryotic organisms with diverse variety of functions by its involvement in mediating protein–protein interactions upon serving as a non-disposable part in multi-protein complexes.

While the mechanisms of the broad range of important functions of RING finger proteins remain enigmatic. Very recently, crucial pieces of evidences have compelled the involvement of the RING-H2 subclass proteins as subunits of the E3 ligases in the ubiquitin proteolytic degradation system [Kamura et al., 1999; Tyers and Rottapel, 1999]. The ubiquitin system is the central regulatory system controlling target protein proteolysis in order to regulate the

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half-life and expression level of proteins participating in cell cycle progression, tumorigenesis, development, and signal transduction pathways [Hershko and Ciechanover, 1998].

RING-H2 subclass proteins including Rbx1/Roc1, SAG/Roc2, and Apc11p are identified as essential components of the distinct superfamily of E3 ubiquitin ligase complexes in control of cell cycle progression: namely, the Skp-Cdc53-F-box (SCF), the VHL-Elongin C/Elongin B (VCB), and the Anaphase-Promoting Complex (APC) [Tyers and Jorgensen, 2000]. Distinct from the HECT domain type E3 ligases, they are characterized by the catalytic core formation by the interaction between the RING-H2 finger proteins with a cullin domain subunit in this E3 ligase superfamily [Zachariae et al., 1998; Joazeiro and Weissman, 2000]. Also, the integrity of the RING-H2 domain in these E3 ligases is often critical for ubiquitination to occur [Chen et al., 2000; Swaroop et al., 2000]. The cullin family member, cdc53, in the SCF is responsible for recruiting the E2 ubiquitin conjugating enzyme [Patton et al., 1998] while the RING-H2 finger protein, Rbx1, operates to tether the E2 to the complex and activate it [Seol et al., 1999; Skowyra et al., 1999].

The anaphase-promoting complex or cyclosome (APC/C), is one of the cullin/RING type ubiquitin E3 ligases. It regulates important events in cell cycle progression, especially in mitosis. The separation of sister chromatids and the exit from mitosis to G1 phase; by targeting the degradation of regulatory factors including securin and mitotic cyclins for degradation by the 26S proteasome through ubiquitination [King et al., 1996; Wolf and Jackson, 1998; Kramer et al., 2000].

The APC/C protein complex is composed of at least 12 subunits in budding yeast and at least 10 subunits in mammals [Zachariae and Nasmyth, 1999; Yamanaka et al., 2000]. Yeast Apc2 is found to be a distant member of a family of proteins called cullins [Zachariae et al., 1998] while Apc11p is found to contain a RING-H2 finger motif. These two subunits present in the APC/C are thought to resemble the homology with that of cdc53 and Rbx1 in SCF E3 ligase for recruiting E2 ubiquitin conjugating enzymes to target regulatory proteins for degradation [Gmachl et al., 2000]. Moreover, Apc11p is found to be capable of mediating E1 activating enzyme and E2 conjugating enzyme for the ubiquitination of protein substrates in vitro. Both budding yeast cell viability and E3 ligase activity are shown to be dependent on the integrity of the RING-H2 finger in Apc11p [Leverson et al., 2000].

We report in this study the identification, sequence analysis, tissue distribution, chromosomal mapping, and subcellular localization of ANAPC11, the human homolog of yeast Apc11p isolated during a large-scale partial sequencing of human liver cancer cDNA library.

MATERIALS AND METHODS

Cloning and Sequence Analysis

A human liver cancer cDNA library was constructed unidirectionally in λZAP expression vector and partial sequencing of the cDNA clones isolated was conducted. Briefly, eluted phage plaques were subjected to polymerase chain reaction (PCR) in a pair of primers flanking the cloning sites of the vector. Cycle sequencing of PCR products was carried out using fluorescein-labelled T3 primers and Thermosequenase cycle sequencing kit (Amersham Pharmacia Biotech, Ohio). The sequencing products were run and analyzed in an automated sequencer (Li-Cor). Sequence analysis and comparisons against the GenBank/EMBL nucleotide and protein databases were performed using the BLAST network server at the National Center for Biotechnology Information (NCBI).

One of the full-length cDNA clones exhibited DNA sequence similar to the RING-H2 finger family. PCR of the ANAPC11 cDNA was performed using a pair of primers flanking the open reading frame (ORF) of ANAPC11 (Forward: 5′-TAG GGC GAA TTC ATG AAG GTG AAG ATT AAG TGC TGG A-3′ and Reverse: 5′-TAG GGC GTC GAC TCA CTC CTT GAA CTT CCA TTC CTG-3′). Both primers have an end clamp (TAG GGC), which facilitate cleavage by restriction enzymes. An *Eco*RI site and a *Sal*I site were introduced in the forward and reverse primers respectively. After digestion with *Eco*RI and *Sal*I restriction endonucleases, the PCR fragment was cloned into the plasmid pGBKT7.

Chromosomal Mapping-Somatic Cell Hybrid Analysis

PCR was applied to a panel of 24 somatic cell hybrid DNAs plus three control DNAs including human, Chinese hamster and mouse (Human/

Rodent Somatic Cell Hybrids Mapping Panel 2; National Institute of General Medicine Service, Coriell Cell Repositories). Forward primer used for PCR is located at the 3′ end of the coding region of ANAPC11 and the reverse primer located at the 3′ untranslated region having sequences: 5′-GCA TTG CAT CCT CAA GTG G-3′ and 5′-GAG TCT CAG GAT GCC CCT C-3′ respectively. PCR reaction mixtures (25 μl each) containing 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTP, 0.25 μM of each primer, 0.25 U *Taq* polymerase and 0.5 ng hybrid DNA were prepared. Cycling conditions were as follows: 94°C for 5 min, 40 cycles of 95°C (36 s), 55°C (36 s), 72°C (20 s) and a final extension of 72°C for 10 min. Twelve microliter of each PCR product were analyzed on a 2% agarose gel and visualized under ultraviolet light by staining with ethidium bromide.

Subcellular Localization Analysis

To investigate the subcellular localization of ANAPC11, GFP-ANAPC11 hybrid was constructed by cloning ANAPC11 cDNA into the pEGFP-C1 vector (Clontech, Palo Alto, CA). To facilitate the cleavage by restriction endonucleases, both PCR primers have an end clamp (TAG GGC) with which a *Bgl*II site and a *HinD*III site were added to the forward and reverse primers respectively. (Forward: 5′-TAG GGC AGA TCT ATG AAG GTG AAG ATT AAG TGC TGG A-3′ and Reverse: 5′-TAG GGC AAG CTT TCA CTC CTT GAA CTT CCA TTC CTG-3′). The GFP-ANAPC11 hybrid construct and pEGFP-C1 vectors were used to transiently transfect with lipofectamine into non-cancerous liver cell (AML 12), cancerous liver cell line (HepG2), and normal mouse skeletal muscle myoblasts (C2C12). After 24 h transfection, the subcellular localization of the GFP-only and GFP-ANAPC11 hybrid proteins was examined using a fluorescent microscope.

Northern Blot Analysis

Human Multiple Tissue Northern Blot (MTN) filter (Clontech), which contains normalized poly(A) RNA of various human tissues, was hybridized with the ANAPC11 cDNA labelled by the random priming method using α-^{32}P dCTP (Amersham Pharmacia Biotech). The hybridization was carried out in ExpressHyb Hybridization solution (Clontech) at 68°C, and the membrane was washed in 2X SSC with 0.05% SDS at 42°C and in 0.1X SSC with 0.1% SDS again at 42°C. Autoradiography was performed at −70°C for 120 h.

Dot Blot Analysis

ANAPC11 cDNA labelled by the random priming method using α-^{32}P dCTP (Amersham Pharmacia Biotech) was used to hybridise the Multiple Tissue Expression (MTE) Array RNA master blot (Clontech). The array contains normalized loading of each poly(A) RNA from different human normal and fetal tissues and the cancer cell lines along with several controls. The loading of RNA has been normalized against eight different house keeping genes that belonged to different functional classes and showed minimal variations. Prehybridization was carried out at 65°C with 1.5 mg sheared salmon testes DNA added to ExpressHyb solution (Clontech). Hybridization was performed at 65°C for 6 h followed by washing in 2X SSC with 1% SDS at 65°C for four times, and in 0.1X SSC with 0.5% SDS at 55°C for two times. Autoradiography was performed at −70°C for varying lengths of time ranging from 6 h to 6 days. Dot intensities were quantified and analyzed by scanning densitometry with desitometer (Molecular Dynamics).

RESULTS

Sequence Analysis of ANAPC11

In attempt to establish a resource for searching tumor markers of human hepatocellular carcinoma (HCC), our group had initiated a project on partial cDNA sequencing of a human normal liver and a human liver cancer cDNA libraries. One of the cDNA clones called td156, exhibited high DNA sequence similarity with that of the RING-H2 finger domain protein family as well as the yeast Apc11p protein. The gene encoded by this cDNA is therefore named ANAPC11, the anaphase promoting complex subunit 11 (yeast APC11 homolog). The sequence of ANAPC11 has been submitted to GenBank/EMBL Data Libraries with assigned accession number AF247565 and the gene name and gene symbol approved by HUGO/GDB Nomenclature Committee. ANAPC11 cDNA insert is 514 base pairs in length with the start and the stop codons located at positions 71 and 325, respectively. An atypical polyadenylation signal (ATTAAA), instead of the conventional polyadenylation signal (AATAAA), is found at nucleotide number 496–501 (Fig. 1). The unu-

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1    G GAG TTT CGT CAT GTT GGC CAG GCC CAT TTG AGA TCT TTG AAG ATA      46

47  TCC TCA ACG TGA GGC TCT GCT GCC ATG AAG GTG AAG ATT AAG TGC TGG      94
1                                   M   K   V   K   I   K   C   W       8

95  AAC GGC GTG GCC ACT TGG CTC TGG GTG GCC AAC GAT GAG AAC TGT GGC     142
9   N   G   V   A   T   W   L   W   V   A   N   D   E   N   C   G      24

143 ATC TGC AGG ATG GCA TTT AAC GGA TGC TGC CCT GAC TGC AAG GTG CCC     190
25  I   C   R   M   A   F   N   G   C   C   P   D   C   K   V   P      40

191 GGC GAC GAC TGC CCG CTG GTG TGG GGC CAG TGC TCC CAC TGC TTC CAC     238
41  G   D   D   C   P   L   V   W   G   Q   C   S   H   C   F   H      56

239 ATG CAT TGC ATC CTC AAG TGG CTG CAC GCA CAG CAG GTG CAG CAG CAC     286
57  M   H   C   I   L   K   W   L   H   A   Q   Q   V   Q   Q   H      72

287 TGC CCC ATG TGC CGC CAG GAA TGG AAG TTC AAG GAG TGA GGC CCG ACC     334
73  C   P   M   C   R   Q   E   W   K   F   K   E   *                  85

335 TGG CTC TCG CTG GAG GGG CAT CCT GAG ACT CCT TCC TCA TGC TGG CGC     382

383 CGA TGG CTG CTG GGG ACA GCG CCC CTG AGC TGC AAC AAG GTG GAA ACA     430

431 AGG GCT GGA GCT GCG TTT GTT TTG CCA TCA CTA TGT TGA CAC TTT TAT     478

479 CCA ATA AGT GAA AAC TCA TTA AAC TAC TCA AAT CTT                     514
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Fig. 1. The cDNA and predicted amino acid sequences of ANAPC11. Sequence data of ANAPC11 have been deposited with GenBank/EMBL Data Libraries under the Accession Number AF247565. Gene symbol and gene name have been approved by the HUGO/GDB Nomenclature Committee. Conserved cysteines/histidines residues in the RING-H2 finger domain are shown in bold.

sual polyadenylation signal is also present in some mammalian genes such as Von Hippel-Lindau binding protein (VBP1) [Brinke et al., 1997].

The ORF of ANAPC11 encodes 84 amino acids (predicted molecular weight = 9.84 kDa) (Fig. 1). The protein sequence has an excess of basic residues (7 lysyl, 5 histidyl, and 2 arginyl) over acidic ones (4 aspartyl and 3 glutamyl). Therefore, the isoelectric point of the predicted protein is 7.62 as determined by the software PROSIS. The protein sequence as predicted from the ANAPC11 cDNA sequence possesses a putative $C_3H_2C_3$ (RING-H_2) zinc RING-H_2 finger domain at the C terminus of the protein while the whole protein contains quite a number of WD repeats which serve as protein–protein interaction sites. Database searches identified ANAPC11 as a highly conserved gene whose *Rattus norvegicus* (Rn), *Mus musculus* (Mm), *Drosophila melanogaster* (Dm), *Caenorhabditis elegans* (Ce), *Arabidopsis thaliana* (At), *Saccharomyces cerevisiae* (Sc), and *Saccharomyces pombe* (Sp) homologs share 98, 98, 73, 48, 73, 44, and 54% protein sequence identities with ANAPC11, respectively (Fig. 2A). Database searches also identified three additional genes Apc11p, Rbx1/Roc1, and Roc2/SAG that are closely related to ANAPC11 [Kamura et al., 1999; Ohta et al., 1999]. ANAPC11 shares overall protein sequence identities of 37, 37 and 44% with Rbx1/Roc1, Roc2/SAG, and Apc11p respectively (Fig. 2B). When the protein sequence of ANAPC11 and its closely related RING-H2 finger domain proteins were aligned, some notable features were observed (Fig. 2). The consensus sequence of the subclass of these RING-H2 finger domains is $CXICRX_6CX_2CX_{6-9}$(D/E)XCX(L/V)(V/A) (W/I)GXCXHXFHX_2CX_3 W(L/V)X_{5-8}CPX(C/D) (The conserved amino acid residues are represented by bold letters). Taken together, ANAPC11 is found to be a human homolog of Apc11p which is a subunit of the APC/C, a member of a distinct superfamily of E3 ubiquitin ligase complexes including the SCF, the VCB, and the APC [Tyers and Rottapel, 1999; Tyers and Willems, 1999].

Chromosomal Mapping-Somatic Cell Hybrid Analysis

To determine the chromosomal location of ANAPC11, PCR was applied to a monochromosomal NIGMS Human/Rodent Somatic Cell Hybrid Mapping Panel 2. Human genomic DNA (Human line IMR91), the somatic cell hybrid NA10498 (retained human chromosome 17) yielded a band of about 125 bp, same size as the single band in the human control DNA lane (Fig. 3). Therefore, the ANAPC11 gene is located at chromosome 17. An approximately 200 bp PCR product was seen in lanes 1, 16, 17, 20, and 21, with the size corresponding to that of the mouse control DNA lane. It may suggest that a mouse homolog with larger transcript

Panel A

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                    ....|....|....|....|....|....|....|....|....|....|....|....|....|.
                        5        15        25        35        45        55        65
ANAPC11 (Hs)        MKVKIKCWNGVATWLWVANDE- ---------- -------NCG ICRMAFNGCCPDCKVPGDDC
H32307 (Rn)         MKVKIKCWNGVATWLWVANDE- ---------- -------NCG ICRMAFNGCCPDCKVPGDDC
NP_079665 (Mm)      MKVKIKCWNGVATWLWVANDE- ---------- -------NCG ICRMAFNGCCPDCKVPGDDC
lmg gene (Dm)       MKVTIKSWTGVATWRWIANDE- ---------- -------NCG ICRMSFESTCPECALPGDDC
CAA86328 (Ce)    -TRMSITVKKLHVCGEWKWLQGGED ---------- -------TCG ICRMEFESACNMCKFPGDDC
AAF26089 (At)       -------------- ---------- ---------- ----------MAFDGCCPDCKLPGDDC
Apc11p (Sc)         MKVKINEVHSVFAWSWHIPSTS DEDAANNDPI GNDEDEDVCG ICRASYNGTCPSCKFPGDQC
CAB52266 (Sp)       MKVKILRYHAIANWTWDTPKD- ---------- ------DVCG ICRVPFDGCCPQCTSPGDNC

                    ....|....|.....| ....|....| ....|....| ....|....| ....|....|
                            75          85          95         105         115
ANAPC11 (Hs)        PLVWGQCSHCFHMHCILKWLHAQQVQ--QHCPMCRQEWKFKE
H32307 (Rn)         PLVWGQCSHCFHMHCILKWLNAQQVQ--QHCPMCRQEWKFKE
NP_079665 (Mm)      PLVWGQCSHCFHMHCILKWLNAQQVQ--QHCPMCRQEWKFKE
lmg gene (Dm)       PLVWGVCSHCFHMHCIVKWLNLQPLN--KQCPMCRQSWKFMMENSSNDAQPHLTGLPPLPKSLSA
CAA86328 (Ce)       PLVLGICRHAFHRHCIDKWIAAPTNQPRAQCPLCRQDWTIVE
AAF26089 (At)       PLIWGACNHAFHLHCILKWVNSQTSQ--AHCPMCRREWQFKE
Apc11p (Sc)         PLVIGLCHHNFHDHCIYRWLDTPTSK--GLCPMCRQTFQLQKGLAINDAHVQKFVEIVSRRREEM
CAB52266 (Sp)       PIVWGKCKHIFHAHCIQNWLATSGSQ--GQCPMDRQTFVVADSTNE-KSETQ
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Panel B

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ANAPC11  1                              MKVKIKCWNGVATWLW----------------VANDEN---      22
Rbx1     20                             KRFEVKKWNAVALWAW----------------DIVVDN---      41
Roc2     28                             KMFSLKKWNAVAMWSW----------------DVECDT---      49
Apc11p   1                              MKVKINEVHSVFAWSWHIPSTSDEDAANNDPIGNDEDEDV       40

ANAPC11  23   CGICRMAFNGCCPDCKV-----PGDDCPLVWGQCSHCFHMHCILKWLHA--QQVQQHCPMCRQEWKFKE   84
Rbx1     42   CAICRNHIMDLCIECQANQASATSEECTVAWGVCNHAFHFHCISRWLKTRQV-----CPLDNREWEFQK  102
Roc2     50   CAICRVQVMDACLRCQA---ENKQEDCVVVWGECNHSFHNCCMSLWVKQNNR-----CPLCQQDWVVQR  110
Apc11p   41   CGICRASYNGTCPSCKF-----PGDQCPLVIGLCHHNFHDHCIYRWLDTPT--SKGLCPMCRQTFQLQK  102
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Fig. 2. **A:** Alignments of ANAPC11 coding sequences from *Homo sapiens* (Hs), *R. norvegicus* (Rn) (Accession No.: H32307), *M. musculus* (Mm) (Accession No.: NP_079665), *D. melanogaster* (Dm) (Accession No.: AAF52694), *C. elegans* (Ce) (Accession No.: CAA86328), *A. thaliana (At)* (Accession No.: AAF26089), *S. cerevisiae* (Sc) (Accession No.: NP_010276), and *S. pombe* (Sp) (Accession No.: CAB52266). **B:** Multiple sequence alignments of ANAPC11, Rbx1/Roc1 [Kamura et al., 1999], Roc2/Sag [Ohta et al., 1999] and Apc11p (Accession No.: NP_010276) protein sequences. A bar '—' represents a space inserted for maximising the homology. The consensus amino acid residues are in bold face.

size exists. When the DNA sequence of ANAPC11 was searched against the database of expressed sequence tags (dbEST), it matched a human EST sequence with the GenBank Accession Number D60576. Searching data of RH consortium gene map 99-chromosome 17 map on the Genome Database (http://www.gdb.org), amplimers stSG27175 were

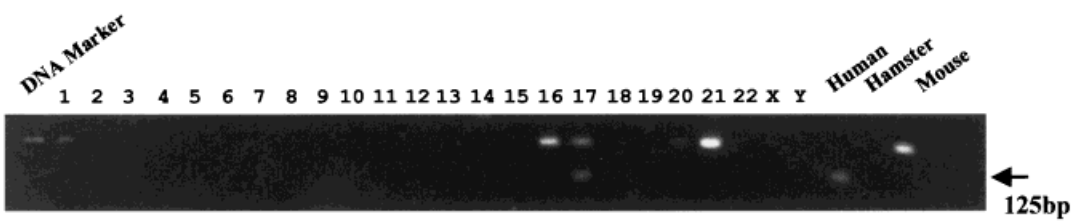

Fig. 3. PCR screening of a panel of 24 somatic cell hybrid DNAs (human/rodent somatic cell hybrids mapping panel 2-version 3; National Institute of General Medicine Service, Coriell Cell Repositories). Labels on the top of each gel lanes denote human chromosome(s) retained in the somatic cell hybrids. Human, hamster, and mouse DNA are included as control and DNA size marker is 100 bp DNA Ladder Plus (MBI Fermentas).

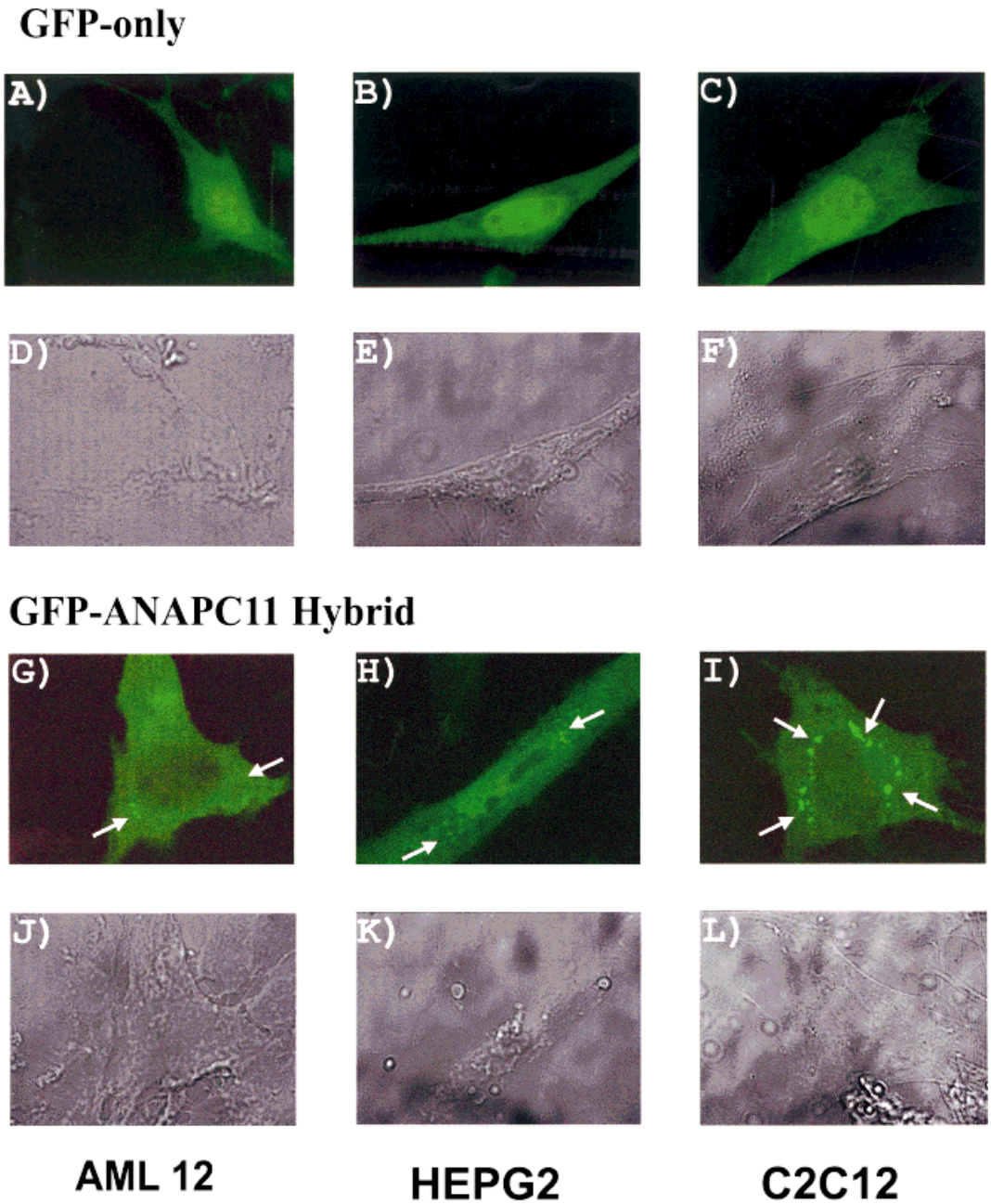

Fig. 4. Subcellular localization of GFP and GFP-ANAPC11 hybrid proteins in mammalian cell lines. GFP-only (**A–F**) and GFP-ANAPC11 hybrid constructs (**G–L**) were used to transiently transfect different cell lines. AML12 hepatocytes were transfected with hybrid constructs shown in (A, D, G, J). Human hepatoma (HepG2] cells were transfected with hybrid constructs shown in (B, E, H, K). Normal mouse skeletal muscle myoblasts (C2C12] were transfected with hybrid constructs shown in (C, F, I, L). The subcellular distribution of the GFP hybrid proteins was examined using a fluorescent microscope. The arrows denote the discrete accumulation of GFP-ANAPC11 hybrid in granular sub-cellular structures.

used to map the EST clone (D60576) and it was placed 542.22 cR (LOD = 0.04) from the top of the Chr17 linkage group by RH Transcript Mapping Consortium, hence, it is located at human chromosome 17, q25 region.

Subcellular Localization Analysis

To analyze the subcellular localization of ANAPC11, GFP-ANAPC11 hybrid was constructed by cloning into pEGFP-C1 vector

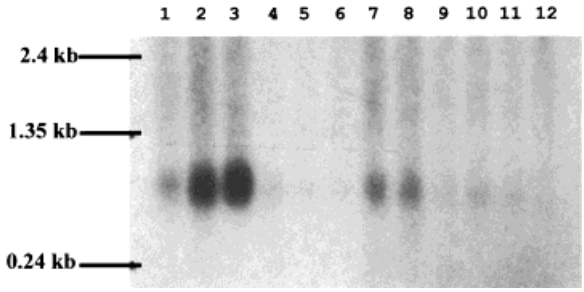

Fig. 5. Northern hybridization of ANAPC11 in human tissues. Key of the **lanes: 1**, brain; **2**, heart; **3**, skeletal muscle; **4**, colon; **5**, thymus; **6**, spleen; **7**, kidney; **8**, liver; **9**, small intestine; **10**, placenta; **11**, lung; and **12**, peripheral blood leukocyte.

(Clontech). These GFP-ANAPC11 hybrid construct and pEGFP-C1 vectors were used to transiently transfect non-cancerous liver cells, AML 12, cancerous liver cells, HepG2, and normal mouse skeletal muscle myoblasts (C2C12). The subcellular localization of the GFP-only and GFP-ANAPC11 hybrid proteins was then examined using a fluorescent microscope. While the GFP protein is distributed diffusely in both cytoplasm and nucleus in all the cell lines. GFP-ANAPC11 hybrid protein is also found in both cytoplasm and nucleus, but with discrete accumulation in granular subcellular structures throughout cytoplasm and in the peri-nuclear region in all cell lines studied (Fig. 4).

Expression of ANAPC11 in Human Normal, Fetal, and Cancer Tissues

To examine the normal tissue distribution of ANAPC11 in human, ANAPC11 cDNA probe was used to hybridize a Northern blot containing normalized poly(A) RNA from a variety of human tissues. It was shown that skeletal muscle and heart have the highest signals. Moderate signal could be seen in brain, kidney, and liver, while low but detectable signals were found in colon, thymus, spleen, small intestine, placenta, lung, and peripheral blood leukocyte (Fig. 5).

In order to obtain the insight on the functions of ANAPC11 and its mRNA expression levels in different tissues, developmental stages, and cancer development, the MTE array with poly(A) RNA samples with various tissues including the neural, cardiovascular, digestive system, other peripheral tissues, different fetal tissues, and different cancer cell lines were employed. In Figure 6, the poly(A) RNA samples were normalized to the mRNA expression levels of eight housekeeping genes that belonged to different functional classes and showed minimal variations. After densitometry measurement, relative abundance of ANAPC11 expression can be compared. In accordance with the Northern Blot analysis, ANAPC11 is found to express ubiquitously in every tissues, with highest expression in skeletal muscle, heart, testis, liver, and kidney and moderate expression in secretary glands.

In comparing the developmental changes between fetal and adult normal tissues, most adult tissues showed two or three-fold increase from the corresponding fetal tissues. Except that the adult lung showed a three-fold decrease in relative intensity compared to that of fetal lung, suggesting that ANAPC11 may have a regulatory role in lung development. For the study on ANAPC11 expression levels in cancer development, leukemia cell lines including K-562 and MOLT-4 showed significant increase (7-fold) in ANAPC11 expression compared to that of normal adult peripheral leukocyte. Lung carcinoma cell line A549 also showed a two-fold increase in ANAPC11 expression compared to that of normal adult lung. Therefore, ANAPC11 may have tentative function in carcinogenesis of these tissues.

DISCUSSION

We have isolated the human homolog of Apc11p, ANAPC11, which is a subunit in the APC/C that contains a RING-H2 finger domain and shares sequence similarity with a recently identified subclass of RING-H2 finger proteins that serve as E3 ligases in the ubiquitin proteolytic degradation system, critical for the regulation of cellular process especially in cell cycle regulatory control [Kamura et al., 1999; Ohta et al., 1999].

Database searches with ANAPC11 sequence had identified three similar RING-H2 finger domain proteins: Apc11p, Rbx1/Roc1, and Roc2/SAG. These RING-H2 finger domain proteins define a new superfamily of E3 ubiquitin ligase complexes: the SCF, the VCB, and the APC/C. They play a crucial role in providing target specificity and selectivity on the recognition and conjugation of substrates by a small basic highly conserved protein, ubiquitin, through a cascade of ubiquitin transferase enzymes, E1→E2→E3, to tag protein substrates for targeting and degradation by the 26S proteasome [Hershko

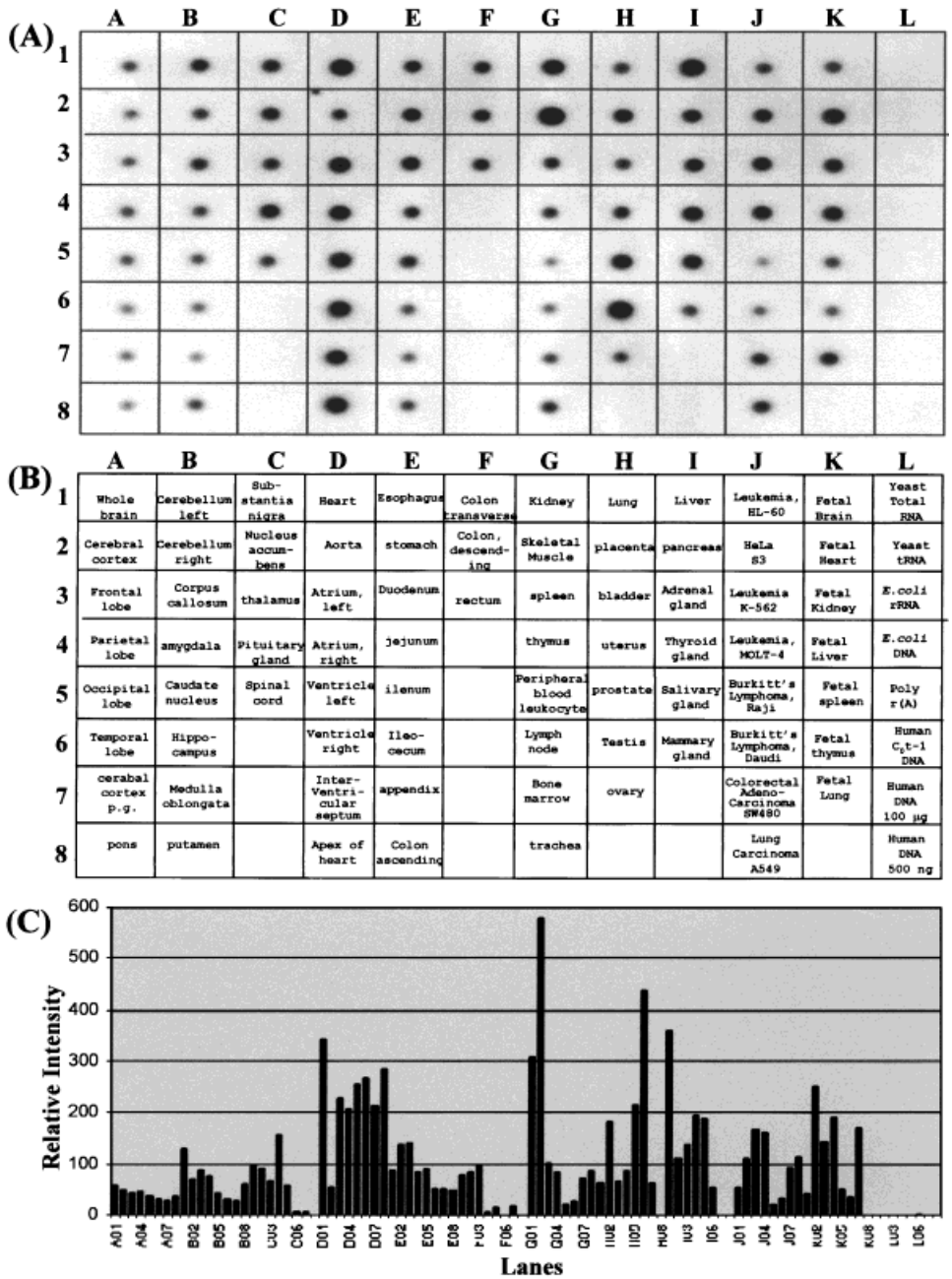

Fig. 6. **A:** ANAPC11 mRNA expression in different tissues on the MTE Array (Clontech) RNA Dot Blot. **B:** Diagram showing the type and position of poly(A) RNA dotted on the positively charged nylon membrane. **C:** Intensity of the spots determined by densitometry scanning of the film, data analyzed, and displayed in arbitrary units of relative intensity.

and Ciechanover, 1998; Tyers and Jorgensen, 2000].

ANAPC11 shares not only sequence similarity with these proteins but also the tissue distribution and the subcellular localization. They are ubiquitously expressed in human tissues. ANAPC11 as well as Roc2/SAG showed high expression level in heart, skeletal muscle, and testis [Duan et al., 1999] while Rbx1 is found to be prevalent in mouse genital tract [Perin et al., 1999]. The expression of these RING-H2 fingers in tissues that require high energy supply for cellular movements (muscle cell contraction and sperm cell mobility) may be accounted by the anti-apoptotic activity of RING finger proteins including Roc2/SAG, the

IAP family members, SIAH, Livin, survivin, etc. [Adida et al., 1998; Sun, 1999; Germani et al., 2000; Yang and Li, 2000; Kasof and Gomes, 2001]. Moreover, RING-H2 fingers in both Roc2/SAG and Apc11p are essential to yeast cell viability [Leverson et al., 2000; Swaroop et al., 2000].

Roc2/SAG is found to be distributed diffusely throughout the cytoplasm and nucleus of the cell [Duan et al., 1999]. Similarly, ANAPC11 is shown in this study to be localized in both cytoplasm and nucleus, but also with discrete accumulation in granular structures, which are suspected to be resulted by the aggregation of the 26S proteasome in the cytoplasm and perinuclear region. Being a subunit of the APC/C, ANAPC11 also showed similar tissue distributions with other subunits in most tissues, suggesting the variation in expression levels of APC/C subunits is interdependent and that APC11 is functional and crucial in all tissues [Jorgensen et al., 2001].

During analysis of the liver EST sequencing database, we found that the abundance of ANAPC11 cDNA in liver cancer and normal liver libraries are 0.088 (3 out of 3,411) and 0% (0 out of 4,717) respectively. Thus, ANAPC11 is a possible candidate gene being up-regulated in hepatocellular carcinoma. ANAPC11 was predicted to be located at the distal end of the long arm of chromosome 17 (near 17q25 region). Another RING finger, survivin which is an anti-apoptosis gene expressed in cancer and lymphoma [Ambrosini et al., 1997, 1998] is also mapped to the same region. Interestingly, this region of chromosome 17 is often deleted in several human malignancies including ovarian cancer [Dion et al., 2000], breast cancer [Fukino et al., 1999], oesophageal squamous cell carcinoma [Mandard et al., 2000], desmoplastic infantile ganglioglioma [Park et al., 1996], primary cutaneous melanoma [Bastian et al., 1998], and alveolar soft-part sarcoma [Joyama et al., 1999].

In addition, mutation in one of the subunits of E3 ubiquitin ligase is found to associate with tumorgenesis. Von Hippel-Lindau (VHL) protein is a subunit of an E3 ubiquitin ligase complex of VCB. Mutation of the VHL gene predisposes individuals to a wide range of tumors: namely, renal cell carcinoma, pheochromocytoma, cerebellar hemangioblastomas, and retinal angioma [Tyers and Rottapel, 1999]. The proper functions of the APC/C is also essential to cells because missegregation of sister chromatids will result in aneuploidy in daughter cells that is frequently associated with cancers [Lengauer et al., 1998; Nasmyth, 1999]. Coherently, ANAPC11 is shown to be differentially expressed in different cancer cell lines and its expression is identified to be up-regulated in leukemia and lung cancer cell lines compared to the corresponding normal tissue expression. Therefore, ANAPC11 may be involved in the process of tumor development.

Conceivably, the possible roles of ANAPC11 in cell cycle progression as E3 ligases to sensitize the degradation of cell cycle regulatory factors, the developmental regulatory role, the up-regulation in malignancies, and the identification of the protein interacting partners will be interesting areas for future investigations.

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