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CLONING OF A cDNA WHICH ENCODES A NOVEL UBIQUITIN-LIKE PROTEIN+

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SUMMARY: The cloning of a mouse cDNA, *Nedd-8*, which encodes a small novel protein of 81 amino acid residues with a relative molecular mass of 9 kDa, is described. The putative *Nedd-8* product is approximately 60% identical to ubiquitin protein. The 0.6 kb mRNA for *Nedd-8* gene can be detected in various mouse tissues and cell lines derived from various sources. *Nedd-8* probe hybridizes to genomic DNA from various vertebrate species as well as yeast, indicating high degree of interspecies conservation. These data suggest that like ubiquitin, the product of this gene may play some essential role in eukaryotic cellular metabolism.

Ubiquitin is one of the most evolutionarily conserved eukaryotic protein and as the name implies, expressed in all cells (reviewed in ref. 1). This small, 76 amino acid protein has been implicated in variety of cellular functions such as ATP dependent proteolysis of abnormal or short lived normal proteins (2), stress related response (3-7), apoptosis (8), DNA repair (9), cell cycle control (10, 11), chromatin organization (12) and ribosomal biogenesis (13). Three classes of ubiquitin genes have been described (reviewed in ref. 1). Class I and class II genes code for a monomeric ubiquitin fused at its carboxyl terminus to ribosomal proteins of 52 and 76-81 amino acids respectively, while class III genes code for polyubiquitin precursors that contain tandem repeats of the 76 amino acid ubiquitin polypeptide followed by a single additional carboxyl terminal amino acid. In addition to these ubiquitin genes, several ubiquitin-like genes have been identified recently from various organisms (14-19). Some of these, such as those encoded by the genomes of bovine diarrhoea viruses CP1 (14) and Osloss (14), and baculovirus (15) are remarkably similar to

+The sequence reported in this paper has been deposited in the DDBJ, GenBank and EMBL databases under accession number D10918.

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ubiquitin. Others encode proteins with more limited homology to ubiquitin, such as *Gdx*, a gene on the human X chromosome which encodes a 157 amino acid polypeptide, of which amino terminal 74 share 43% identity with ubiquitin (16) and *BAT3*, a gene from the human major histocompatibility complex class III encoding a fusion protein with an amino terminus which bears 35% identity with ubiquitin (17). Significantly, the *Gdx* and *BAT3* encoded proteins lack the cleavage site for ubiquitin hydrolase (1).The 15 kDa β-interferon induced protein UCRP, contains two ubiquitin like domains (18,19) and conjugates to cellular proteins (20). Because ubiquitins from various organisms are remarkably conserved (only three amino acid difference between yeast and human ubiquitins), the significant divergence of ubiquitin-like proteins suggests that they may be involved in some specialized ubiquitin related functions such as specific protein-protein interactions.

In a previous study, by subtraction cloning, we had identified several partial cDNA clones whose corresponding genes are expressed at high levels in embryonic mouse brain (21). We report here that one of these cDNA encodes a novel ubiquitin-like protein which is widely expressed in various tissues and cell lines and shows high degree of evolutionary conservation.

MATERIALS AND METHODS

Cloning of Nedd-8

The original *Nedd-8* clone 2D1 isolated from a mouse neural precursor cell (NPC) cDNA library contained an insert of 0.55 kb (21). Using this insert we rescreened the neural precursor cell cDNA library (21) and a commercial cDNA library prepared from mouse embryonal carcinoma cell line PCC4 (Stratagene), using standard procedures (22). A total of five positive clones were isolated, all of which contained inserts similar in length to the original 2D1 clone. The cDNA inserts from these clones were sequenced from both strands using Sequenase (USB). Sequence analyses were performed using the Wisconsin Genetic Computer Group program package. Homology searches were performed using FASTA and TFASTA programs(23).

Northern and Southern analyses

Following cell lines were used for RNA isolation: mouse fibroblast NIH/3T3 (24), mouse embryonal carcinoma P19 (25), F9 (26) and PCC4 (27), mouse neuroblastoma N18 (28), rat astrocytoma C6 (29), rat pheochromocytoma PC12 (30), human neuroblastoma GOTO (31) and SK-N-AS (32), human SV40-transformed mammary epithelium HBL-100 (33) and human fibrosarcoma HT1080 (34). Poly A^+ RNA were isolated by one cycle of oligo-dT cellulose absorption using a kit (Fast Track, Invitrogen) according to the instructions supplied by the manufacturer. For adult mouse tissue northern and interspecies Southern analyses, commercially available blots (Clonetech) were used. Hybridizations were carried out using standard protocols (22).

RESULTS

Nedd-8 cDNA cloning

Several independent clones isolated from two separate cDNA libraries contained similar inserts of approximately 0.55 kb in length which is close to the size of the

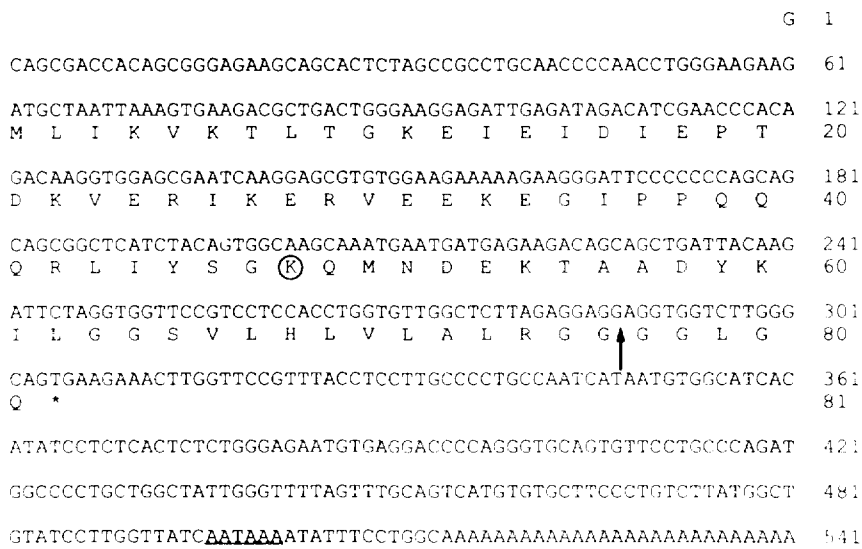

Figure 1. Nucleotide and deduced amino acid sequence of *Nedd-8* cDNA. The nucleotide sequence was obtained from both strands. Amino acids are shown in single letter codes. The lysine residue at position 48 is encircled and the polyadenylation signal AATAAA is underlined. The putative cleavage site for ubiquitin carboxyl terminal hydrolase is indicated by an arrow.

Nedd-8 mRNA (0.6 kb) detected in northern blot analysis (see below) indicating the clones represent near full length sequence of *Nedd-8* cDNA. The complete nucleotide sequence of the *Nedd-8* cDNA consisted of 514 nucleotides (excluding the 3' poly A stretch) (Figure 1). The sequence contains a single long reading frame which is open at its 5' end. The open reading frame, from the first in-frame methionine to the termination codon, can encode a putative protein of 81 amino acid residues and an estimated molecular mass of 9 kDa. The 3' end of the cDNA contains a poly A stretch (17 to 28 nucleotides long in different clones) preceded by an upstream polyadenylation signal AATAAA (Figure 1).

Putative Nedd-8 product is homologous to ubiquitin

Search for related sequences in the nucleotide and protein databases using FASTA and TFASTA programs revealed that *Nedd-8* is approximately 60% identical to ubiquitin, both at nucleotide and amino acid levels. Further alignment of the two protein sequences using Bestfit program indicated that they share 57.8% identity and 78.9% similarity (Figure 2). The most notable difference between the two proteins is the presence of five additional amino acids at the carboxyl terminus of *Nedd-8*. The carboxyl terminal glycine residue of ubiquitin participates in ubiquitin protein conjugation after precursor processing by ubiquitin carboxyl terminal hydrolases to generate the monomeric ubiquitin (reviewed in ref. 1). The carboxyl terminal sequence of *Nedd-8* is very similar to the ubiquitin and it is likely that *Nedd-8* polypeptide also undergoes processing to remove the extra sequence at the

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Nedd-8      MLIKVKTLTGKEIEIDIEPTDKVERIKERV  30
            |  ||||||:|:::|:::|.:::::|:::
Ubiquitin   MQIFVKTLTGKTITLDVEPSDSIDNVKQKI  30

                        *
Nedd-8      EEKEGIPPQQQRLIYSGKQMNDEKTAADYK  60
            ::  |||||:|||||:|||:::|:|| :||:
Ubiquitin   QDKEGIPPDQQRLIFAGKQLEDGRTLSDYN  60

Nedd-8      ILGGSVLHLVLALRGGGGLGQ  81
            |  :|:|||||  ':|:|
Ubiquitin   IQKESTLHLVLRLRGG  76
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Figure 2. Alignment of the putative *Nedd-8* protein with ubiquitin. The two sequences were aligned using Bestfit program. The residues identical between the two protein sequences are indicated by a vertical bar (|), while conservative changes indicated by vertical double dots (:). The lysine residue at position 48 is indicated by an asterisk.

carboxyl terminus. The lysine at position 48 of ubiquitin is responsible for self ubiquitination (35) and importantly the corresponding residue is also conserved in *Nedd-8* encoded protein (Figures 1, 2).

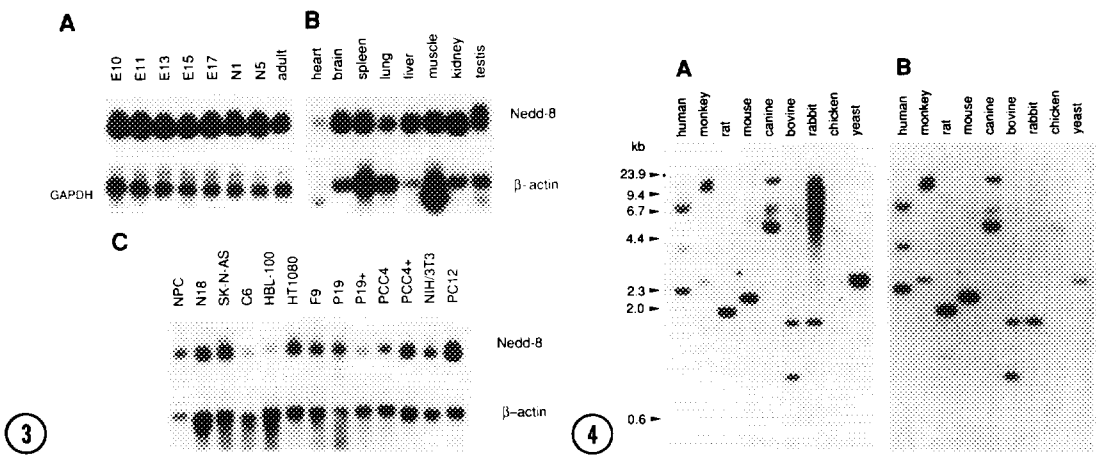

Figure 3. Northern blot analysis of *Nedd-8* expression in embryonic mouse brain **(A)**, adult mouse tissues **(B)**, and various cell lines **(C)**. Poly A^+ RNA (1-2 μg) from indicated sources were hybridized to the full length insert from clone 2D1 (*Nedd-8* probe). Same blots were sequentially hybridized with *Nedd-8* probe and either glyceraldehyde 3-phosphate dehydrogenase (GAPDH) **(A)** or β-actin **(B,C)** probes (see reference 37 and 38 for the details of the probes and hybridization conditions). Where indicated (+), P19 and PCC4 cells were treated with 1μM retinoic acid for two days. In **(A)**, E10-E17 and N1-N5 represent post-coital embryonic and post-natal days, respectively.

Figure 4. Interspecies genomic Southern blot analysis of *Nedd-8*. A commercially obtained interspecies Zoo-blot (Clonetech) was hybridized with *Nedd-8* probe at 65°C in a buffer containing 6xSSC, 5xDenhardt's solution, 0.1% SDS and 100 μg/ml of sheared and denatured salmon sperm DNA (22). The final washes were carried out in 0.5xSSC, 0.1% SDS either at 65°C **(A)** or 70°C **(B)**. Each lane contains 10μg of *Eco* RI digested chromosomal DNA from indicated species.

Expression of Nedd-8 mRNA

Ubiquitin, as the name suggests, is expressed in all cell types (1). To check if this is also true for *Nedd-8*, we analyzed poly A^+ RNA isolated from embryonic mouse brain at various stages of development (Figure 3A), adult mouse tissues (Figure 3B) and several mouse, rat and human cell lines of diverse origins (Figure 3C) by northern blot hybridization. Under experimental conditions used here (final wash in 0.5xSSC, 0.1% SDS at 65°C), no cross hybridization to ubiquitin mRNA was detected. The 0.6 kb *Nedd-8* mRNA was present in all samples analyzed, although the expression levels were somewhat variable.

Interspecies conservation of Nedd-8 gene

From the northern blot analysis described above, it was clear that the mouse *Nedd-8* cDNA probe hybridizes with the rat and human mRNA. To further check the interspecies conservation of *Nedd-8* gene, we carried out Southern hybridization analysis of *Eco*RI digested genomic DNA from various organisms (Figure 4). The *Nedd-8* probe hybridized to DNA from all species examined (human, monkey, rat, mouse, dog, cow, rabbit, chicken and yeast) (Figure 4A) indicating high degree of sequence conservation among both vertebrates and yeast. Even under more stringent washing conditions (0.5xSSC, 0.1% SDS at 70°C) fainter but clear signals for hybridization to yeast DNA can be seen (Figure 4B).

DISCUSSION

In the present communication we have described the isolation of a cDNA which encodes a novel ubiquitin-like protein. The 5' end of the open reading frame derived from *Nedd-8* sequence is open and our attempts to clone longer cDNA by either library screening or 5' RACE (36) have so far been unsuccessful. The open reading frame from the first in-frame methionine to the termination codon, can encode a polypeptide of 81 amino acid residues consisting of a single complete ubiquitin-like unit. Moreover, the sequence upstream of the methionine does not share any significant homology with the sequences in the databases. Taken together, these observations suggest that the *Nedd-8* cDNA sequence reported here contains complete coding region. The predicted *Nedd-8* polypeptide is five amino acid residues longer than ubiquitin. The sequence conservation between *Nedd-8* and ubiquitin in the putative carboxyl terminal proteolytic cleavage site, suggests that this additional sequence may be removed during processing. This would result in an active ubiquitin-like protein which could interact with other cellular proteins. At least in one case, a ubiquitin-like protein has been shown to conjugate with other cellular proteins (20).

Strong apparent interspecies conservation of *Nedd-8* gene and the widespread distribution of its mRNA point to its role in some essential cellular function. Strong

structural homology of *Nedd-8* gene product with ubiquitin indicates a role similar to ubiquitin. On the other hand, its significant divergence from ubiquitin suggests that it may participate in a more specific ubiquitin related function.

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