Sequence Analysis and Chromosomal Localization of Human Cap Z

CONSERVED RESIDUES WITHIN THE ACTIN-BINDING DOMAIN MAY LINK CAP Z TO GELSOLIN/SEVERIN AND PROFILIN PROTEIN FAMILIES*

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From a human retinal cDNA library, we have isolated cDNAs that are homologs for the α and β subunits of chicken Cap Z. The derived human α subunit shares 95% amino acid identity with the chicken α2 subunit; the β subunit is 99% identical to the chicken subunit residues 1-243. The remaining portion of the human β subunit (244-272) diverges significantly with only 8 out of 29 C-terminal amino acids conserved between the two species. This lack of conservation is of particular interest because the chicken C terminus contains an actin-binding domain. Cosedimentation assays with F-actin show that human Cap Z binds actin with an affinity equal that of chicken Cap Z. These results point to the eight shared amino acids as critical for actin binding, three of which are regularly spaced leucines. Three apolar residues and one outside the region of divergence align well with those residues of the actin-binding α-helix proposed for gelsolin segment 1. The apolar residues as well as three polar amino acids are also conserved in other capping, capping and severing, and monomer-binding proteins. Amino acid substitutions in the chicken β subunit of the two most highly conserved leucines result in significant decreases in F-actin binding activity. The human α2 gene (CAPZA2) has been mapped to chromosome 7 position q31.2-q31.3 and the β gene (CAPZB) to chromosome 1 region p36.1.

Cap Z has been identified in chicken as a nonsevering, barbed-end actin-binding protein composed of α and β subunits. In chicken, two cDNAs have been isolated for the α subunit. The α1 and α2 isoforms, which share 95% identity, are the products of two separate genes (1,2); one gene is believed to be responsible for the β subunit expression (3).

By capping the barbed end of actin filaments, Cap Z regulates the growth of the actin filament at the barbed end (4). The actin-binding domains of the protein have been mapped by genetic manipulation of the respective subunit genes. Deletion mutations have shown that the C-terminal 55 amino acids of the α1 subunit (5), and the C-terminal 15 amino acids of the β subunit are necessary for binding to F-actin in vitro (6). When expressed as a glutathione S-transferase fusion protein, the C-terminal 25 residues of the β subunit are sufficient to bind monomeric actin (6).

For comparative studies and as a step toward identifying human mutations, we have isolated and sequenced cDNAs encoding an α and a β subunit for human Cap Z and mapped each subunit gene to its chromosome. We also have expressed the subunits and determined the binding affinity of the human heterodimer to F-actin. Within the divergent β subunit C termini of the Cap Z and other homologous capping proteins, we have identified several conserved residues. We provide the first evidence that many of these conserved amino acids from the Cap Z family of actin-capping proteins align with similar amino acids in actin-capping/severing and monomer binding families of proteins. To confirm the functional importance of these conserved residues, we have made amino acid substitutions for two of the most conserved residues in the β domain of chicken Cap Z and studied the F-actin binding characteristics of the heterodimers (α1/β mutated β) in actin cosedimentation and binding assays.

MATERIALS AND METHODS

Screening of Retinal cDNA Library—A human retinal cDNA library constructed in λgt10 (kindly provided by Dr. Jeremy Nathans) (7) was screened with cDNAs of the chicken α and β subunits. For each of the two screens, approximately 180,000 plaques were transferred onto nitrocellulose, and the filters were prepared for hybridization using standard procedures (8). Hybridizing plaques were isolated and purified by limiting dilution and rescreening.

Subcloning into pBS and pGEM—Phage DNA was isolated from plate lysates using standard procedures (8). After digesting the purified phage DNA with EcoRI, the cDNA insert was gel purified. The resulting cDNAs, which ranged in size from 1-3 kb, were subcloned into pBluescript KS+ (pBS, Stratagene) and sequenced using the dideoxy chain termination method (9). One clone containing the full-length α subunit cDNA (pHαA-1, 1.6 kb) and two clones with the complete β subunit cDNA (pHβA-2, 1.0 kb and pHβA-3, 2.2 kb) were sequenced on both strands.

α and β cDNAs were transferred into pGEM (Promega) in order to synthesize RNA for in vitro translation. To enhance the level of expression from these constructs, an EcoRI/NcoI fragment containing the 5′-untranslated region (UTR) from the immediate early gene, 475 (10), was included. Using the forward primer (5′-CCAGAAGGACCATGGCGGATT27-3′) and reverse primer (5′-AAAATCTTAGATGCAAGTCTTAA30-CCG) for the α subunit and the forward primer (5′-CCAGAAGGACCATGGCGGATT-3′) for the β subunit.
GAGACGCCATCATGAGTG7-3) and the reverse primer (5'-AAATCTAGAGTATGAGCCTGTCG3'-3) for the β subunit and the templates pH4-1 and pH4-3, cDNAs for each subunit were amplified. In addition, the sequences encoding the initiating methionines for each subunit were modified to include an NcoI or the compatible site, BspHI; an XbaI site was created at the 3' end of each cDNA (added restriction sites are highlighted in the primers). By digesting the PCR fragment with either NcoI or BspHI and XbaI, the cDNAs were inserted into the pGEM vector behind the 475 5'-UTR. The cDNAs were sequenced again to check for any nucleotide changes created by PCR amplification.

Construction of Substitution Mutations—Substitution mutations of Leu262 and Leu266 were created in the chicken reverse primer (5'-GTCCTATGCTAAAGCCTCGGT3'-3) for the β subunit and by PCR using the template pGem-β. In the synthesis of two β subunit mutations, restriction enzyme sites were introduced into the nucleotide sequence coding for Glu261-Leu262. To accomplish the modifications, two C-terminal PCR fragments were generated for each restriction site. The 5' PCR fragments were synthesized using a forward primer containing a KpnI site (5'-GGTCTAGACCATGAA3'-3) and a reverse primer with either the restriction site Eco47III (5'-TGGTACCAGTCTCCCTGTG3'-3) or HindII (5'-TGGTACCAGTCTCCCTGTG3'-3). The forward primers used for the 3' PCR fragments were the complement of the reverse primers used in synthesis of the 5' PCR fragments; the reverse primer was the TpI (DNA polymerase) sequence found in pGEM 3' to the multidicing site. After synthesis, the 5' PCR products were digested with KpnI and either Eco47III or HindII and the 3' PCR fragments were digested with either Eco47III or HindII and XbaI. The 5' and 3' fragments were ligated together at the new restriction site and substituted for the wild type KpnI-XbaI fragment containing the β C-terminal domain in pGEM-β. When the Leu266 encoding sequence was mutated to encode an Eco47III site, Leu266 was changed to Arg266; when altered to a HindII site, Ala266 was generated.

Alteration of Leu262 was achieved using a similar strategy. A forward primer (5'-AGAGGACGCTCTCCAGTNGNAGCGGACCG3'-3) and reverse primer (5'-TGAGGACGCTCTCCAGTNGNAGCGGACCG3'-3) were made which changed the β subunit nucleotides 783 and 786, creating a SacI restriction site. These substitutions maintained Glu261-Leu262. To alter the amino acid at position 266, degenerate nucleotides were included at positions 796 and 797 (underlined) in the forward primer. Similar to the previous constructs, a 5' PCR fragment was synthesized using the KpnI-containing forward primer and the SacI-containing reverse primer; a 3' PCR fragment was made using the SacI forward primer (with degenerate nucleotides) and TpI, primer. The fragments were then digested with the appropriate restriction enzymes and ligated together at the SacI site. The resulting KpnI-XbaI fragment was used to replace the wild type sequence in pGEM-β. The nucleotide changes at positions 796 and 797 of these constructs resulted in several amino acid substitutions at Leu266.

Analysis of Sequence—Nonredundant nucleotide sequence data bases were searched for sequences homologous to human Cap Z using the BLASTN program from the National Center for Biotechnology Information (Bethesda, MD) using the BLAST network service.

Chromosomal Localization—Using primers designed from the 5'-UTR of the cDNAs, the human α2 and β subunit genes were localized to two different chromosomal loci by PCR. The primers for the α2 subunit screening were 5'-AGATTTCGACCTGACG3'- (forward) and 5'-GTTAGTATTTCCCTCGTTG3'- (reverse); those for the β were 5'-GAGCTCTTCTTTGGAACGAGGAGTG3'- (forward) and 5'-TCTTTGGAACGAGGAGTG3'- (reverse). Using the recommended conditions for AmpliTaq (Perkin-Elmer), 100 ng of DNA from each sample of the NIGMS human-rodent somatic cell hybrid mapping panel 2 (11) were assayed. Thirty cycles of amplification were performed under the following conditions: 1-min denaturation at 94°C, 2-min annealing at the primer-specific temperature (52–55°C for α primers and 60°C for β primers), and 2-min extension at 72°C.

The localization of CAPZA2 to chromosome 7 was further refined by Southern and PCR analysis of a panel of 17 human-rodent somatic cell hybrids (12, 13) and a chromosome 7-specific yeast artificial chromosome (YAC) library (14). The human α2 cDNA was used as a probe in the hybridization experiments; PCR products were generated using the 3'-UTR primers to human α2 DNA.

The CAPZB gene was regionally localized on chromosome 1 by fluorescence in situ hybridization (FISH) analysis according to established protocols (15–17). A 2.2-kb CAPZB cDNA probe (containing the plasmid vector) was biotinylated with the Life Technologies, Inc. BioNick labeling kit (15°C, 1 h) (15). After overnight hybridization and washing, the signals were amplified and detected using published methods (17). The FISH signals and the DAPI-banding patterns were photographed separately. Assignment of the FISH mapping data to a specific chromosomal band was then determined by superimposing the FISH signals on the DAPI-banded chromosomes (15).

In Vivo Transcription/Translation and Chromatography—[35S]-Labeled protein was synthesized in vitro using transcription and translation kits (Promega) as described previously (5). For transcription, 1 μg of each plasmid was used per every 10 μl of reaction mixture and 0.3–0.5 mM mCAP analogue 5'MGppPsG (Strategene) was added. Synthesis was initiated from the SP6 promoter. For each subunit, approximately 8 μl of RNA were translated in a 100-μl reaction solution containing 70 μl of rabbit reticulocyte lysate. A total of 16 μl of RNA (8 μl for each subunit) was used in the translation of the heterodimer. 100 μCi of [35S]methionine (DuPont NEN) were included in these reactions to label the resulting protein. An estimate of the amount of protein synthesized was calculated based on the number of methionines/subunit and trichloroacetic acid-precipitable counts as described previously (5).

For quantitative binding assays and to assess heterodimer formation, samples were gel-filtered on a 1 × 30-cm Superose 12 FPLC column (Pharmacia). The column was equilibrated and run at a rate of
0.5 ml/min in 100 mM KCl, 0.2 mM dithiothreitol, 0.01% NaN₃, 10 mM K₂HPO₄, pH 8.0. Samples were collected in 0.5-ml fractions. Ten microliters from each fraction were counted in scintillation mixture to determine the column profile. Radiolabeled products were electrophoresed on 10% SDS-polyacrylamide gels and visualized by autoradiography. Computer images of the autoradiograms were collected using the Eagle Eye II Still video system (Stratagene) and analyzed by ImageQuant densitometry analysis software (Molecular Dynamics) to determine the quantity of each subunit.

Cosedimentation and Binding—The cosedimentation of the individual subunits and various heterodimers with chicken F-actin was tested by incubating equimolar amounts of the in vitro translated human Cap Z subunits and heterodimer with actin. Each product was assayed with and without excess native chicken Cap Z. The ³⁵S-labeled samples were run on a 10% SDS-polyacrylamide gel and visualized by autoradiography.

To calculate the binding of the intact and mutated proteins to F-actin, the same assay was performed except that gel-filtered heterodimer was used and the concentration of actin was 0.25 mg/ml. After washing the pellet three times in the above buffer minus the 20% sucrose, the amount of labeled material in the pellet was determined by scintillation counting. Kᵦ values of the proteins were calculated using the LIGAND program of Munson and Rodbard (18) as modified by McPherson (19).

RESULTS

Nucleotide and Predicted Amino Acid Sequence—Several human cDNAs were isolated from a human retinal library for both the Cap Z α and β subunits. In the α screening, 38 hybridizing plaques were identified; 15 plaques hybridized with the β probe. For each subunit, the cDNA inserts from 5 plaques chosen randomly were isolated, cloned and sequenced. All of the cloned cDNAs contained varying amounts of the same α or β subunit sequence. GenBank™ accession numbers for the human α₂ and β cDNAs are U03269 and U03271, respectively. At the time of submission to the databank, sequence identity was detected with three databank entries (nos. T11326, D12250, and M26658), none of which contained complete subunit cDNA sequence nor had been identified as Cap Z.

Analysis of the human α nucleotide sequence showed that it shared 90% identity with the chicken α₂ and 80% identity with α₁. The predicted amino acid sequence (Fig. 1A) revealed only 14 differences between the human and chicken α₂ subunits out of 286 residues.

The human β sequence from base 1-729 shared 87% nucleotide identity with the chicken β nucleotide sequence resulting in 99% amino acid identity between chicken and human. The remaining sequence of the human β subunit markedly differed from the chicken β (Fig. 1B). Sequence alignments revealed that a 113 nucleotide segment that corresponds to the C-terminal domain of the chicken β subunit is absent in the human cDNA. As a substitute, 90 nucleotides that are similar (95% nucleotide identity) to a region considered 3'-untranslated region in chicken have become coding region for the C terminus of the human β subunit. The human and chicken C termini differ in length and share only 27% identity.
Actin Cosedimentation and Binding Studies with Human Cap Z—The divergence of the human \( \beta \) sequence in a putative actin-binding domain of the chicken subunit raised the question of whether the human protein retained actin-binding activity. To address this question, radiolabeled human \( \alpha \) and \( \beta \) subunits produced by in vitro translation were examined for their ability to form a heterodimeric protein and bind to actin. In vitro translated, \( ^{35} \text{S} \)-labeled human \( \alpha \) (Fig. 2, top) and \( \beta \) (center) subunits chromatographed on a Superose 12 FPLC column showed a major peak at fraction 41. When both \( \alpha \) and \( \beta \) were translated together, two peaks were seen (bottom). One peak eluted from the column at a position similar to that of the individual subunits; the other eluted at fraction 37, in a position identical to heterodimeric native chicken Cap Z. An aliquot of fraction 37 electrophoresed on a SDS-polyacrylamide gel and autoradiographed showed approximately equimolar amounts of each subunit when analyzed by densitometry and adjusted for \( ^{35} \text{S} \) incorporation. Upon incubation and sedimentation of the individual subunits when analyzed by densitometry and adjusted for \( ^{35} \text{S} \) incorporation. Upon incubation and sedimentation of the individual subunits and heterodimer with F-actin, only the heterodimer pelleted appreciably with actin (Fig. 3). Both sedimentations could be reduced by the addition of excess native chicken Cap Z. Binding studies performed with the gel-filtered heterodimers (repeated >10 times) showed a \( K_d \) of approximately \( 7 \times 10^{-11} \text{ M} \). Sequence Comparison between the C-terminal Domain of Human and Chicken Cap Z \( \beta \) Subunits and Other Barbed End, Actin-Binding Proteins—The ability of the human protein to bind actin despite its weak similarity to the presumed actin-binding domain in the chicken protein prompted further examination of the C-terminal amino acid sequence. Aligned C-terminal domains of human (29 amino acids) and chicken (34 amino acids) revealed 8 amino acids that were identical (Fig. 4). Within the Cap Z family of actin-binding proteins, these 8 residues are conserved with the exception of the glutamine (Q) at position 270. A few of the intervening amino acids also appear to be maintained or conservatively substituted as shown in the consensus sequence for Cap Z and homologs. The most obvious feature of the comparison was an array of three, regularly spaced leucines that aligned with positions 258, 262, and 266 of human and chicken. When compared to other functionally distinct actin-binding proteins, such as the gelsolin (20), gelsolin-related (21–25), and monomer-binding (26) proteins, we found a similar pattern of apolar residues that aligned with the leucine-rich array of Cap Z (positions 258, 262, 266, and perhaps 265). In addition, an apolar amino acid immediately adjacent to the region of divergence in the human and chicken C termini (aligning with human position 243) was conserved. Polar amino acids that were conserved in several actin-binding proteins (20, 26) are aligned with the Cap Z \( \beta \) sequences. Boxes indicate identical or conservatively substituted amino acids among the different families of proteins. The numbers of the first and last residues of the aligned sequence are given. Those apolar amino acids that are believed to be involved in actin binding of gelsolin S1 are shaded. The 5 amino acids thought to be conserved in several actin-binding proteins are indicated in the alignment by +.
heterodimers suggesting that a global disruption of protein folding was not responsible for the loss in cosedimentation. These complexes eluted in fractions 37 and 38 (Fig. 6), similar to wild type Cap Z. To quantitate the relative amounts of α and β subunits, the purified complexes were electrophoresed on SDS-polyacrylamide gels and autoradiographed. Densitometry scans of the autoradiograms (Fig. 7, A–C) revealed ratios of α and β subunits (Fig. 7C) which were very close to the idealized ratio of the labeled subunits (α:β, 36:64%) determined by the number of [35S]methionines incorporated into each subunit. These complexes containing the altered β subunits were then tested for their ability to bind to actin. The results of the cosedimentation experiments (Fig. 5) correlated with calculated $K_D$ values determined from the binding assays (Fig. 7D). Both show that actin binding of Cap Z is more affected when the leucine at position 262 is changed than the leucine at position 266. At each position, some amino acid substitutions show greater alteration in actin binding than others. A nonconservative substitution at either leucine with a basic residue significantly reduced binding; replacements with small apolar amino acids, such as Gly and Ala, also showed marked reductions.

Chromosomal Localization of the Human Cap Z α and β Subunit Genes—Chromosomal localizations of CAPZA2 and CAPZB were determined by PCR screening of NIGMS human-rat somatic cell hybrid mapping panel 2 (11). PCR products of the expected size for the α2 (343 bp) and β (231 bp) subunit genes were only amplified from the hybrid cell lines containing human chromosome 7 and human chromosome 1, respectively (data not shown).

To refine the localization of CAPZA2, a panel of 17 human-rat somatic cell hybrids containing defined regions of human chromosome 7 (12, 13) was analyzed by the described PCR assay. The results clearly indicated that the CAPZA2 gene mapped to the 7q31.2-q32 region (data not shown). The PCR primers were then used to screen a human chromosome 7-specific YAC library (14) and four YACs (HSC7E4, HSC7E1394, HSC7E1420, and HSC7E1440) were identified (Fig. 8). These YAC clones were previously localized to 7q31.2-q31.3 and shown to be part of a set of overlapping clones linking the MET proto-oncogene to the cystic fibrosis transmembrane regulator gene (CFTR) (12). HSC7E4 (600 kb), HSC7E1394 (330 kb), and HSC7E1420 (360 kb) were found to contain both MET and CAPZA2; therefore, the two genes are separated by a maximum distance of 330 kb. The results of these mapping studies suggest a physical order of the following reference DNA markers for the 7q31.2-q31.3 region: cen-MET-CAPZA2-D7S122-WNT2-D7S633-(D7S677-CFTR)-tel.

The CAPZB gene was regionally localized to chromosome 1 region p36.1 by FISH analysis (Fig. 9). The efficiency of hybridization of the cDNA clone to metaphase chromosome preparations was approximately 60%. DAPI banding was used to assign the signal specifically to chromosome 1, which was in agreement with the PCR mapping data. A total of 10 mitotic figures were photographed, and the results indicated that CAPZB mapped to 1p36.1 (Fig. 9).

**DISCUSSION**

**Sequence Comparison between Human and Chicken Cap Z**—Our results show that the majority of human Cap Z is over 95% identical to the α2β1 heterodimer of chicken Cap Z. One of the striking findings in this study was the decrease in conservation to 27% in the C-terminal domain of the β subunit. The nucleotide sequence shows that a 113-nucleotide segment present in the chicken β cDNA is not represented in the human cDNA. This results in a region considered 3'-UTR in chicken becoming the coding sequence for the human β subunit C terminus. A chicken β subunit cDNA with a C terminus similar to our human clone has been identified recently and given the name β2 (27). Although the genomic structure is not yet known...
for either the chicken or human $\beta$ genes, the most probable explanation is that the missing portion of coding sequence has been alternatively spliced from the $\beta$ subunit RNA.

Candidate human $\alpha_1$ and $\beta_1$ homologues were not isolated in this study. Their lack of representation may be due to the source of the cDNA libraries screened. Both may be absent or in low abundance in the retina; alternatively, these isoforms may be absent in the human.

Lack of Homology in an Actin Binding Domain—Previous sequence comparisons between capping proteins of lower eukaryotes and chicken Cap Z have demonstrated a strong conservation of primary structure except in the C-terminal region of the $\beta$ subunit (6). It was therefore surprising that an actin-binding domain was mapped to this region of the chicken $\beta$
subunit. When expressed as a fusion protein, the C-terminal
region alone did not cap actin filaments but instead bound
monomeric actin with a $K_D$ of 325 nM. Data base searches
between this sequence and monomer binding or barbed-end
capping proteins revealed no significant similarities (6).

Similarly, the human heterodimer with its differing $\beta$ C-
terminal sequence binds actin with an affinity equal to or
greater than the two isoforms of chicken Cap Z (5). As with the
chicken protein, the individual human subunits required one
another for high affinity binding. The similarity in binding yet
lack of sequence identity with the chicken actin-binding do-
main pointed to the eight conserved residues as being poten-
tially critical for actin binding.

Conserved Amino Acids in the C-terminal Domain—

Conserved in those few conserved amino acids is a leucine-rich
array that is present in variable form in other Cap Z homologs,
KXXK$^{\text{L262}}$XXE/DE$^{\text{L266}}$XXX/K/R. This sequence is found
within the C-terminal 25 amino acids of the chicken $\beta$ subunit
presumed to contain an actin-binding site. Our experiments
show that amino acid substitutions at Leu$^{\text{262}}$ and Leu$^{\text{266}}$ with
either polar or small apolar residues greatly reduced the cap-
ping protein's ability to bind actin without impairing the ability
of the subunit to interact with the heterologous subunit. The
leucines at positions 262 and 266 of Cap Z align with leucines
contained within a highly conserved five amino acid peptide
described in the gelsolin and profilin protein families and in a
wide range of actin-binding proteins (28, 29).

A recent structural study of gelsolin S1 fragment (30) com-
plicated with actin suggests that a patch of apolar residues
inserts between subdomains 1 and 3 of actin and is rimmed
with hydrogen bonding residues. From this model, several of
the residues of the highly conserved five amino acid sequence
in gelsolin S1 as well as several amino acids surrounding this
sequence are involved in this interaction (30). The leucine-rich
motif (including position 243) identified in the Cap Z family of
proteins aligns well with the apolar residues of gelsolin that are
proposed to interact with actin. Like the corresponding region
of gelsolin, this sequence in Cap Z is predicted to be $\alpha$ helical.

Previous studies have failed to show strong homologies be-
tween Cap Z and other actin-binding proteins. A weak homol-
ogy exists between chicken Cap Z $\beta$ subunit residues 113–152
and gCAP39 (Cap G) residues 116–160 and, to a lesser extent,
gelsolin residues 117–159 (23). Within this segment of gelsolin,
a short stretch of basic amino acids is present which is thought
to be important in phosphoinositide binding (31). The findings
of the present study provide the first evidence of a potential
functional link between the actin-binding domains of Cap Z and
the gelsolin/severin and profilin families. The fact that a

![Fig. 8. A YAC contig surrounding the gene at 7q31.2-q31.3. Twelve YAC clones (A) spanning the MET, CAPZA2, WNT2, and CFTR genes (B) are shown. The location of the DNA markers used to define the YAC contig are shown (C); the shaded boxes represent the minimal region to which the DNA markers could be assigned. The exon specific primers (exons 1, 5, 10, 15, 20, and 24) are from the CFTR gene. The other DNA markers have been described (12).](image)

![Fig. 9. Regional localization CAPZB to 1p36.1. An idiogram of chromosome 1 summarizes the FISH analysis using the $\beta$ subunit cDNA. Each dot represents the localization of double fluorescent signals on banded chromosome 1.](image)
common motif has been preserved among three distinct families of actin-binding proteins is interesting, and suggests that binding sites in all three families may have evolved from a common source.

Chromosomal Localization of Human Genes—At this time, it is unclear if any genetic disease might result from mutations in the human Cap Z genes. Interstitial deletions involving the q21–32 region of chromosome 7 are commonly observed chromosome abnormalities (32). The clinical presentation of these and other analyses. Likewise, the localization of CAPZB to chromosome 1p36.1 will also provide a valuable DNA marker for similar physical mapping studies of chromosome 1.

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Note Added in Proof—Several cDNAs, including expressed sequence tags (ESTs) have been identified recently which may represent partial sequences of a human Cap Z n1 subunit.

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