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Source: Zoological Science, 17(8) : 1095-1099

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.17.1095

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ZOOLOGICAL SCIENCE **17**: 1095–1099 (2000) © 2000 Zoological Society of Japan

A 7.5-kb 3'-Terminal cDNA Sequence of Chicken Skeletal Muscle Nebulin Reveals Its Actin Binding Regions

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ABSTRACT—Nebulin is an approximately 700 kDa filamentous protein in vertebrate skeletal muscle. It binds to the Z line and also binds side-by-side to the entire thin (actin) filament in a sarcomere. Nebulin is currently thought to be a molecular ruler regulating the length of the thin filament to 1 μm. The complete sequence of human skeletal muscle nebulin was determined by Labeit and Kolmerer(1995). Because of its large size, only fragmental sequence information has been available for nebulins other than human skeletal muscle. This paper describes for the first time the sequence of about one third (C terminal region) of chicken skeletal muscle nebulin. It was found that the fundamental structure of human nebulin, consisting of 35 amino acid repeats (modules) plus C terminal serine-rich and SH3 domains linked to the Z line are well conserved with chicken nebulin. Sequence identity ranged from 74 to 91%. There were super-repeats (seven modules), a first linker repeat, simple repeat and a second linker repeat in addition to the Z line binding region as in human nebulin. However, there were 2 fewer modules in the first linker repeat and 6 fewer in the simple repeat in chicken nebulin as compared to human nebulin. Two isoforms of chicken nebulin were sequenced indicating insertion of approximately 6 or 11 modules to a structure similar to that of human nebulin. Recombinant first linker repeats M51~56 were shown to bind to actin using the ELISA technique as well as human nebulin recombinants.

INTRODUCTION

Nebulin is an approximately 700 kDa giant protein of vertebrate skeletal muscle which was discovered by Wang and Williamson (1980). It is localized along the entire thin filament from the Z line to its pointed end (Wang and Wright, 1988; Maruyama *et al*., 1989). Later information on its amino acid sequence has led to the so-called molecular ruler hypothesis that a single nebulin molecule, 1 μm in length, spans the whole length of the thin filament to stabilize it resulting in a discrete unit length of this filament (Kruger *et al*., 1991; Labeit *et al*., 1991; Trinick, 1994; Labeit and Kolmerer, 1995). Immunoelectron microscopy revealed that the C terminus of nebulin binds to the Z line (Wright *et al*., 1993).

The nucleotide sequence of nebulin cDNA derived from human skeletal muscle predicts that nebulin consists of 6669 amino acid residues with a molecular mass of 773 kDa. Human nebulin contains 185 repeats of 35 amino acid residues (modules) and each module has a predicted α-helix structure and contains a central SDXXYK structure, an actin binding region (Labeit *et al*., 1991; Labeit and Kolmerer, 1995). In the I band region of nebulin, seven modules are organized into a super-repeat, which is thought to bind to the 38.5 nm repeating unit of the thin filament containing seven actin molecules, and one troponin/tropomyosin complex. Twenty two super-repeats consisting of 154 modules span 0.85 μm in the thin filament of a sarcomere (Labeit and Kolmerer, 1995).

So far the complete sequence of nebulin has been described with human skeletal muscle (Labeit and Kolmerer, 1995), although fragmental information on the nebulin sequence is also available for rabbit and mouse skeletal muscles (Labeit *et al*., 1991; Muller-Seitz, *et al*., 1993; Zhang *et al*., 1996; Millevoi *et al*., 1998). We have sequenced approximately one third of the nebulin cDNA from chicken breast muscle, corresponding to the C terminal region (2402 amino acid residues) of nebulin (near the Z line region). This paper, albeit not yet providing a complete sequence, will contribute to the understanding of structure and function of nebulin in skeletal muscle.

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MATERIALS AND METHODS

Cloning and sequencing of chicken nebulin cDNA clones

A cDNA library was constructed from adult chicken breast muscle poly $(A)^+$ RNA (Yajima *et al.*, 1996).

A cDNA fragment was obtained as a by-product, when proteins interacting with the N terminal region of chicken skeletal muscle connectin were screened by the yeast two hybrid system (Ohtsuka *et al.* , 1997). This cDNA (N400) turned out to be a fragment of nebulin cDNA. Using the N400 as probe, three successive clones extending in the 3' direction were screened. Both strands of each cDNA clone were sequenced by the dideoxynucleotide chain-termination method with an ALFexpress II DNA sequencer (Amersham Pharmacia Biotech).

Recombinant nebulin fragment

cDNA coding the first linker repeat from P in M51 to the last T in M56 (215 amino acids, MW 24,403) was amplified by PCR. The nebulin cDNA fragment was inserted to a pGEX6p-1 vector (Amersham Pharmacia Biotech) and the GST-tagged linker repeat fragment was

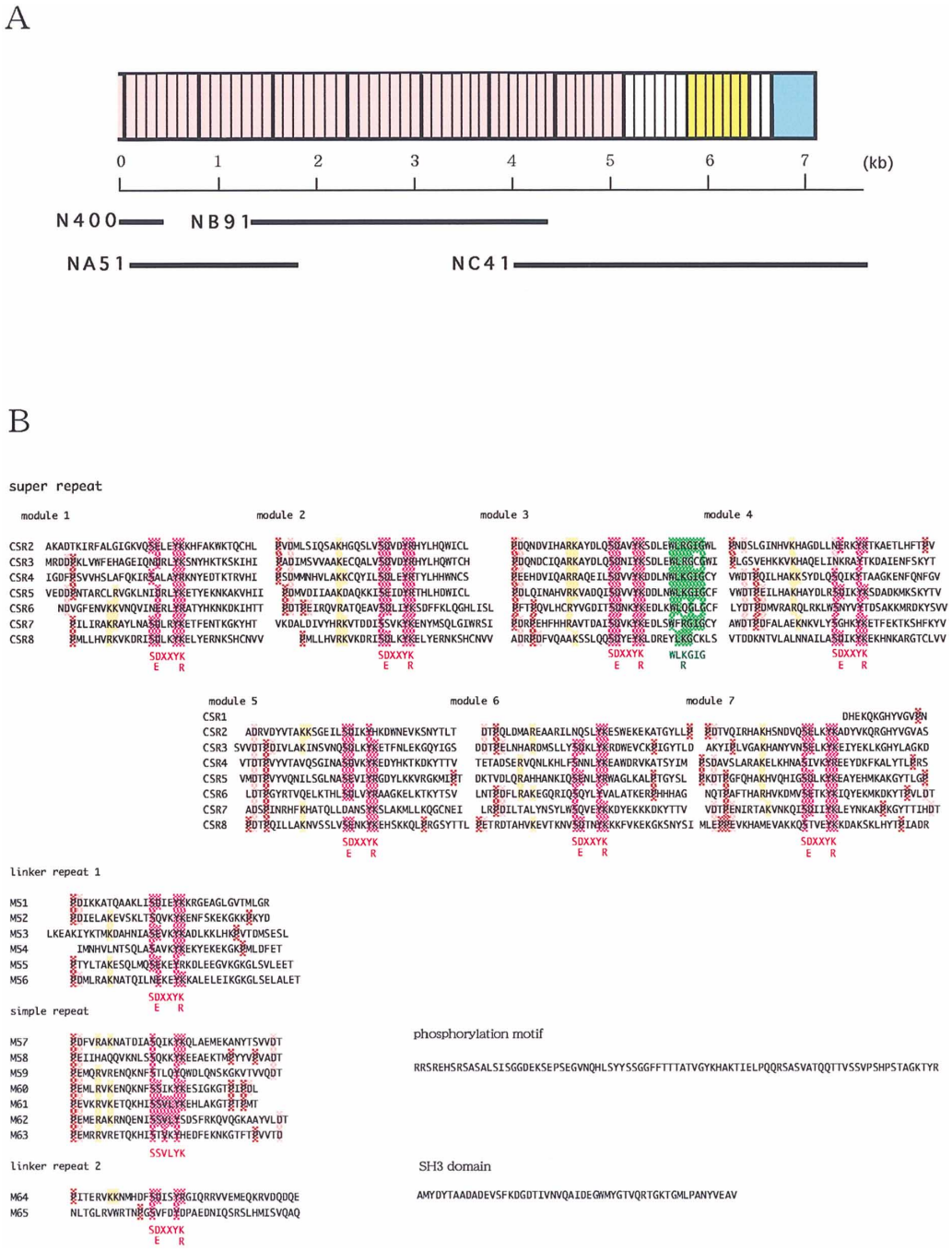


Fig. 1. Sequencing of 7584 bp in the 3' terminal region of chicken skeletal muscle nebulin. A, cloning of nebulin. The nebulin cDNA N400 was extended in 3' direction. Pink boxes, seven-module super-repeats (CSR1-CSR8 of B); white boxes, linker repeats (M51-M56 and M64-M65 of B); yellow boxes, simple repeats (M57-M63 of B); blue box, C terminal region containing phosphorylation motif and SH3 domains; B, deduced amino acid sequence of each portion shown in A. CSR, chicken super-repeat; M, module.

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abundantly expressed in *Escherichia coli* BL21. Cultured cells were disrupted by sonic vibration and the GST-fused linker repeat fragment was adsorbed by Glutathione Sepharose column, GST was cut off by PreScission Proteinase (Amersham Pharmacia Biotech), and then the linker repeat fragment was eluted.

Enzyme-linked Immunosorbent Assay (ELISA)

One hundred μl of nebulin recombinant protein or chicken egg albumin (Sigma, A5503), 10 μg/ml, was coated on 96-well multiplates at 4°C overnight. After the wells were washed with tris-buffered saline (pH 7.2) containing 0.05% Tween 20 and blocked with 1% bovine serum albumin (Sigma) in tris-buffered saline, various amounts of actin purified from rabbit skeletal muscle were added to each well and incubated for 2 hr at room temperature. After washing the wells with tris-buffered saline containing 0.05% Tween 20, anti-α-actin monoclonal antibody (A-2172, Sigma) was added to the wells, followed by treatment with peroxidase-conjugated anti-mouse immunoglobulin (Bio Rad). The interactions were visualized with 0.7 mg/ml orthophenylenediamine in 100 mM citrate buffer, pH4.5. Optical density at 490 nm was recorded with MR580 MicroELISA autoreader (Dynatech).

RESULTS AND DISCUSSION

Sequence of chicken skeletal nebulin

A two hybrid system using cDNA coding the N terminal region of chicken skeletal muscle connectin resulted in isolation of a cDNA clone called N400 (cf. Ohtsuka *et al.* , 1997). Sequencing revealed that N400 had an identity (66.7%) with the sequence of amino acid 4011–4139 of human skeletal muscle nebulin . Walking to the 3' terminal region was carried out using N400 and the clones NA51, NB91 and NC41 were obtained (Fig. 1A). At the end of NC41 there was a stop codon followed by about 400 nucleotides with untranslated region. Therefore, this region was identified with the 3' terminal region of chicken breast muscle nebulin cDNA. Figure 1B shows the sequence of the 2402 deduced amino acids coded by 7584 bp in the 3' terminal region of nebulin cDNA (DDBJ/EMBL/Gen Bank Accession number, AB024330). In the C terminal region of human nebulin, the SH3 domain and serine-rich region (phosphorylation motif) were followed by 35 amino acid repeats (modules), a second linker repeat, simple repeat, first linker repeat and then a super-repeat of seven modules (for terminology, see Labeit and Kolmerer, 1995). This basic structure of human nebulin is strictly conserved in chicken nebulin (Fig. 1A).

Conservative sequences of 35 amino acids in each module of super-repeats in human skeletal nebulin have been repeatedly reported (Labeit *et al.*, 1991; Labeit and Kolmerer, 1995; Wang *et al.*, 1996). As shown in Fig. 1B, each module contained an SDXXYK motif, an actin binding site. Aspartic acid residues in the SDXXYK motif were sometimes replaced by glutamic acid residues and lysine was replaced by arginine. In addition, there was always a unique tyrosine residue in the motif. There was frequently lysine or arginine residue 6~7 amino acids ahead of the serine residue. Around the end toward the N terminus of each module one proline residue was conserved. There was a WLKGIG motif in the third module of each super-repeat in which lysine was frequently replaced by arginine.

The very high identity of each domain of chicken nebulin with human nebulin was as follows: 74% (super-repeat), 77% (first linker), 89% (simple), 91% (second linker), 74% (serin-rich) and 91% (SH3) (Fig. 2). Similarity was 94, 95, 97, 96, 95 and 98%, respectively. However, 2 modules were lacking in the first linker repeat region and 6 modules in the simple repeat region in chicken nebulin as compared to human nebulin. The former deleted portion corresponds to the sequence from the 15th amino acid residue (Y) of M166 to the 18th (I) of M167 and from the 19th (R) of M168 to the 18th (Y) of M169 of human nebulin. The latter corresponds to the sequence

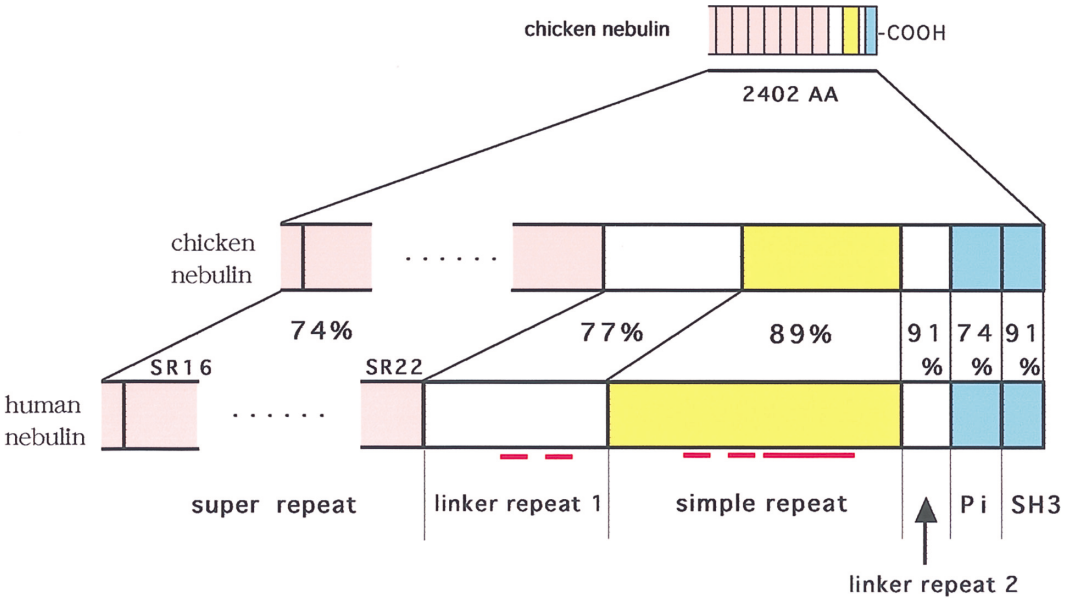


Fig. 2. Comparison of the C terminal region of chicken nebulin with human nebulin. Percentages indicate sequence identity. SR, super-repeat; Pi, serine-rich phosphorylation motif; SH3, SH3 domains. Red lines in linker and simple repeats show deletion region in chicken nebulin.

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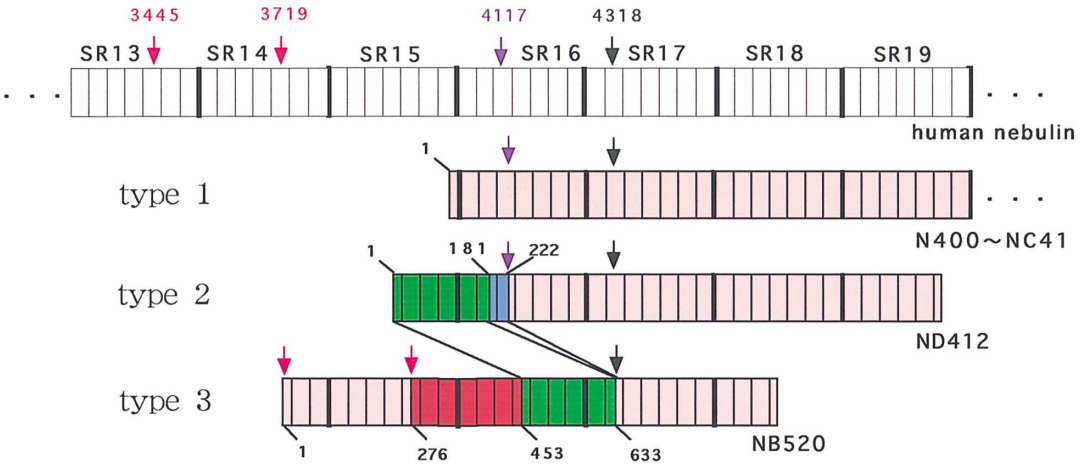


Fig. 3. Isoforms of chicken skeletal muscle nebulin. Isoform type 1 corresponds to human nebulin. Isoform type 2 contains regions of green and blue boxes. Isoform type 3 contains regions of red and green boxes.

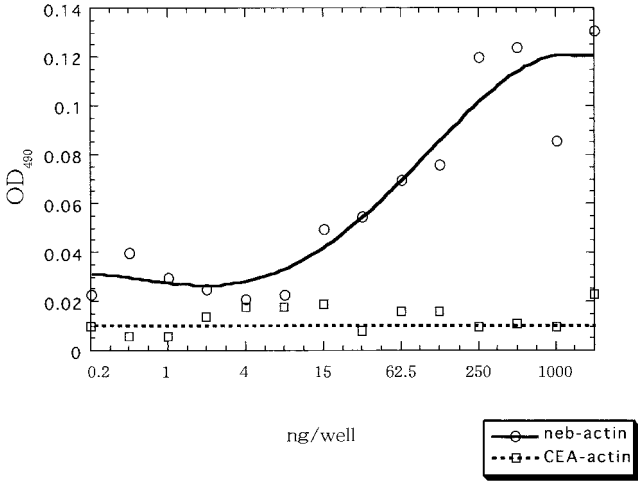


Fig. 4. Binding of recombinant nebulin fragment to actin as revealed by ELISA assay. One microgram of nebulin recombinant protein or chicken egg albumin was added to each well. After a wash with 0.05% Tween 20 saline, blocking with 1% bovine serum albumin, and another wash with saline, various amounts of actin were added. After treatment with anti α-actin monoclonal antibody and then with horseradish peroxidase-labelled antibody, color was developed with orthophenylenediamine (0.7 mg/ml), in 100 mM citrate buffer, pH 4.5. Optical density at 490 nm was measured. neb-actin, nebulin recombinant-actin interactions; CEA-actin, chicken egg albumin-actin interactions.

from the 17th amino acid residue (L) of M174 to the 16th (V) of M175, from the 17th (M) of M176 to the 16th (V) of M177, and from the 1st (P) of M178 to the last (T) of M181 (red lines in Fig. 2). These regional deletions in chicken nebulin cDNA may be related to the fact that the molecular weight of the nebulin is less than that of human nebulin (Kruger *et al.*, 1991), and that the length of the thin filament in chicken skeletal muscle is somewhat shorter than that of human skeletal muscle (Kruger *et al.*, 1991; Walker *et al.*, 1974). Even so these regions are believed to be highly conserved because of their physiologically important function in the binding to the Z line (Labeit and Kolmerer, 1995; Millevoi *et al.*, 1998, Moncman and Wang, 1999).

Worthy of mention also is that there were deletions of 4–5 modules around modules 176–182 in various rabbit skeletal muscle nebulins (Millevoi *et al.*, 1998).

Isoforms of Chicken Skeletal Nebulin

Labeit and Kolmerer (1995) reported that there were at least five isoforms of human skeletal nebulin, all of them different around the mid point of the super-repeat regions.

In the present study, three types of chicken nebulin were found in the upward region of super-repeat 17 (SR17) corresponding to human nebulin (Fig. 3). Type 1 (DDBJ/EMBL/Gen Bank Accession number, AB024330) was very similar to human nebulin with an identity of approximately 70%. This sequence was adopted as that of chicken nebulin (cf. Fig. 1). In type 2 (DDBJ/EMBL/Gen Bank Accession number, AB032421), the amino acid sequence downward from 222 of ND412 (pink) was the same as that of type 1, but that from 221 to 1 had less than 50% identity with human nebulin as compared to 70% in the sequence downward from 222. The sequence from 221 to 181 (blue) was different from that of type 1 with 81% identity to human nebulin isoforms 10b and 10c (Labeit and Kolmerer, 1995). In type 3 (DDBJ/EMBL/Gen Bank Accession number, AB032422), the amino acid sequence downward from 633 of NB520 (pink) was the same as in type 1 with 70% identity to human nebulin. Upward from 632 to 453 (green), the sequence from 180 to 1 of type 2 (green) was inserted followed by a sequence with 50% identity to human nebulin from 452 to 276 (red). The blue region in type 2 was completely deleted, then human nebulin sequence, 3719 to 3445 (red arrows), was linked. The presence of these isoforms suggest alternative splicing of the nebulin gene.

Binding of linker-repeat recombinant

A recombinant polypeptide encoded by cDNA corresponding to the first linker repeat, M51-M56 (Fig. 1B), was expressed in *E. coli* and purified by GST column chromatography. Using the resultant 25 kDa peptide, the binding to actin was investi-

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gated by an ELISA assay and a clear-cut positive result was obtained (Fig. 4). This is consistent with the results of sedimentation experiments using recombinant linker repeat peptide of human nebulin (Pfuhl *et al.*, 1996). Thus, the actin binding property of chicken nebulin is confirmed.

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(Received April 19, 2000 / Accepted May 8, 2000)

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