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Source: Zoological Science, 16(1) : 125-129

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.16.125

ZOOLOGICAL SCIENCE **16**: 125–129 (1999)

The Expression of the Protochordate Homologue of the Proteasome Regulatory Subunit Rpn12 is Transcriptionally and Post-translationally Regulated during Cleavage Stage

Michiko R. Wada[1], Akikazu R. Murakami[1], Hiroshi Kawamura[1], Rinna Nakamori[1], Kimio J. Tanaka[1], Hiroki Nakayama[2] and Takahito Nishikata[1]*

[1]*Department of Biology, Faculty of Science, Konan University, Okamoto, Higashinada-ku, Kobe 658-8501, Japan*
[2]*Laboratory of Molecular Medicine, Yamanouchi Pharmaceutical Co. Ltd., Miyukigaoka, Tsukuba 305-8585, Japan*

ABSTRACT—In order to identify the maternal mRNAs which have important roles in the very early stage of embryogenesis, a *Ciona intestinalis* 64-cell stage cDNA library was subtracted from an unfertilized egg cDNA library. We thereby cloned *Cipros1*, which encodes the protochordate homologue of the proteasome regulatory subunit Rpn12. Neither *Cipros1* mRNA nor Cipros1 protein showed any spatial localization. However, *Cipros1* mRNA was expressed at a level at least five-times higher in unfertilized eggs and about two-times higher in cleavage stage embryos, than in other embryonic stages. In unfertilized eggs, Cipros1 protein was expressed at a level about twice as higher as during the other stages. Moreover, minor, smaller isoforms of Cipros1 were expressed specifically in unfertilized eggs and during early cleavage stages. Since a single *Cipros1* transcript was detected throughout the development, these smaller isoforms might be generated post-translationally.

INTRODUCTION

In ascidian embryos, the earliest known zygotic transcription produces the mRNA coding for the epidermis-specific gene detected at the 8-cell stage (Chiba *et al*., 1998). Muscle-specific structural genes (actin and myosin: Satou *et al*., 1995; Satoh *et al*., 1996) and a muscle specific regulatory gene (bHLH gene: Araki *et al*., 1994; Satoh *et al*., 1996) start to be transcribed zygotically at the 16- to 32-cell stage. The maternally derived mRNAs which are the predominant mRNAs in unfertilized eggs and cleavage stage embryos might have important roles in controlling the zygotic gene expression. In this study, we tried to identify the maternal transcripts which are expressed predominantly in unfertilized eggs and early cleavage stage embryos.

The 26S proteasome is an essential component of the ubiquitin/ATP-dependent proteolytic pathway in eukaryotic cells and is responsible for the degradation of most cellular short-lived regulatory proteins. This degradation pathway is indispensable for the regulation of fundamental cellular activities, such as cell cycle control, cell proliferation and so on (reviewed by Coux *et al*., 1996). The proteolytic core complex, the so-called 20S proteasome, is a cylindrical particle consisting of four rings, each of which is organized from seven homologous, but not identical, α and β subunits (see, for example, Lupas *et al*., 1993). The 26S proteasome is composed of the 20S proteasome and a complex of regulatory subunits (see, for example, Kanayama *et al*., 1992). The regulatory subunit complex has a crucial role in regulating ubiquitin-dependent proteasome activities.

In the ascidian embryo, using a monoclonal antibody against the 20S proteasome, changes in the subcellular localization and activity of the 20S proteasome depending on the mitotic cell cycle were found (Kawahara and Yokosawa, 1992; Kawahara *et al*., 1992). Moreover, 26S proteasome activity is suggested to be regulated through interconversion between the 26S and 20S proteasomes induced by intracellular calcium mobilization (Kawahara and Yokosawa, 1994). Thus, an understanding of the molecular nature of the regulatory subunit complex and its regulation are crucial for understanding the cellular functions of the 26S proteasome in the ascidian embryos.

In this study, we cloned the gene encoding the ascidian homologue (*Cipros1*) of one of the 26S proteasome regulatory subunits, Rpn12 (Finley *et al*., 1998). Northern blot analysis revealed that the *Cipros1* mRNA was predominantly expressed in unfertilized eggs, suggesting some regulation at

* Corresponding author: Tel. +81-78-435-2511; FAX. +81-78-435-2539.

the transcriptional level. Furthermore, Western blotting and immunocytochemistry using a monoclonal antibody against Cipros1 fusion protein revealed three different isoforms of Cipros1 and their regulated expressions. Taken together, the mRNA and protein expression analyses suggest that Cipros1 expression is regulated post-translationally.

MATERIALS AND METHODS

Unfertilized egg-specific subtracted library

Double-stranded cDNAs obtained from poly(A)$^+$ RNAs of *C. intestinalis* unfertilized eggs and 64-cell stage embryos were ligated to the adaptor R (5'-CGGAAACAGCTATGACCATG-3') or P (5'-TGATCGCGTAGTCGATAGTG-3'), respectively. The unfertilized egg (UF)-cDNA library and 64-cell stage embryo (64)-cDNA library were amplified by PCR with P- or R-primer. The 64-library was subtracted from the UF-library according to the method described by Nakayama *et al.* (1996).

Cloning of *Cipros1*

The expression patterns of randomly selected clones from the subtracted library were reexamined using Southern hybridization with the UF- and 64-libraries. One of the isolated clones (#2-5-21) which was expressed predominantly in the UF-library was designated as *Cipros1*. A full length clone of *Cipros1* was obtained by ordinary screening of an oligo-d(T)-primed Uni-ZAP XR (Stratagene, La Jolla, CA, USA) UF-library, and the sequence of the clone was determined (DSQ-1000L; Shimadzu Co., Kyoto, Japan). For Southern and Northern hybridization, a DIG-labeled full-length *Cipros1* DNA probe was used. *In situ* hybridization was performed as described by Satou *et al.* (1995).

Quantitative RT-PCR

The quantitative RT-PCR protocol was described previously (Wada *et al.*, 1998). Primers used in this work were OCipro-F(5'-GTTTATCTTTTCTGCCATCCAC-3') and OCipro-R(5'-CCTGTTTCTTCATTCTGTTTTG-3'). Expression of tubulin was assayed as an internal control for RNA recovery and cDNA synthesis.

Immunological methods

T7-tagged 6xHis-fusion proteins were prepared by the pET expression system 28 (Novagen Inc., Madison, IW, USA). Monoclonal antibody was raised against the Cipros1-fusion protein following the protocol described by Mita-Miyazawa *et al.* (1987). In the immunocytochemical analysis, FITC (fluorescein isothiocyanate)-, AP (alkaline phosphatase)- and HRP (horseradish peroxidase)-conjugated goat anti-mouse IgG+IgM (H+L) (American Qualex, San Clemente, CA, USA) were used for the secondary antibodies. For the Western blotting, dechorionated eggs and embryos were lysed in the Laemmli SDS sample buffer (Laemmli, 1970), and equal amounts of total protein were loaded in each lane.

RESULTS AND DISCUSSION

Molecular cloning of *Cipros1*

In order to isolate the maternal messages which exist predominantly in unfertilized eggs and in early cleavage stage embryos, a 64-cell stage cDNA library of ascidian (*C. intestinalis*) was subtracted from an unfertilized egg cDNA li-

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Cipros1          1:MKEAVTVYQSLKKEFYRKNANLNVCAQHLCKLKIALTGLSF-LP-STQTTPT-LQEFVLA 57
p31              1:------MYEQLKGEWNRKSPNLSKCGEELGRLKLVLLELNF-LP-TTGTKLT-KQQLILA 51
NIN1             1:M-PSLA-ELTKSLSIAFENGDYAACEKLLPPIKIELIKNNLLIPDLSIQNDIYLNDLMIT 58
MTS3             1:M-STL--DL-NHLADLYDRKDWNACKKELLKLKVELAKQNLFVPT-S-DKE---KA-SFA 50
                                       *   *   *  *       *

Cipros1         58:RDVLEIGAQMSIMKRDIPAFERYMSQLKCYYLDYTEDVPESAYKQELLGLNLLCLLAQNR 117
p31             52:RDILEIGAQWSILRKDIPSFERYMAQLKCYYFDYKEQLPESAYMHQLLGLNLLFLLSQNR 111
NIN1            59:KRILEVGALASIQTFNFDSFENYFNQLKPYYFSNNHKLSESDKKSKLISLYLLNLLSQNN 118
MTS3            51:RNVFEYGVLVSIQTCDIESFARYASQVIPFY--HD-SLVPSSRMGLVTGLNLLYLLSENR 107
                      * *   **       *  *  *    *         *        * ** **  *

Cipros1        118:VAEFHTEL--ERLSTNDILNNVYIRHPIAIEQYLMEGNYNK--LFLAKGNVPAESYNYFI 173
p31            112:VAEFHTEL--ERLPAKDIQTNVYIKHPVSLEQYLMEGSYNK--VFLAKGNIPAESYTFFI 167
NIN1           119:TTKFHSELQYLDKHIKNLEDDSLLSYPIKLDRWLMEGSYQKAWDLLQSGSQNISEFDSFT 178
MTS3           108:IAEFHTALESVPDK-SLFERDPYVEWVISLEQNVMEGAFDKVASMIRSCNFPEFSY--FM 164
                     **  *                          ***   *               *

Cipros1        174:DILLGTIRDEIATCIEKSYPRISYTEARRMLYFESENELDQYSKNRDWEL-GAGKY-FCF 231
p31            168:DILLDTIRDEIAGCIEKAYEKILFTEATRILFFNTPKKMTDYAKKRGWVL-GPNNY-YSF 225
NIN1           179:DILKSAIRDEIAKNTELSYDFLPLSNIKALLFFNNEKETEKFALERNWPIVNSKVYFNNQ 238
MTS3           165:KIVMSMVRNEIATCAEKVYSEIPLSNATSLLYLENTKETEKLAEERGWDIRDGVIYFPKE 224
                     *     * ***   *  *          *             * *       *

Cipros1        232:NITKQNEE-TGLVATAELAQQVIKYAKELEMII                        263
p31            226:ASQQQKPE-DTTIPSTELAKQVIEYARQLEMIV                        257
NIN1           239:SK--EKAD---Y--EDEM-MHEEDQKTNI--IEKAMDYAISIENIV          274
MTS3           225:ANALETEDGMLIDEEDELELPPTASKHTISSIRQLLSYTSELEQIV           270
                                 *             *
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Fig. 1. A comparison of the Cipros1 amino acid sequence with those of human p31 (Kominami *et al.*, 1995), yeast NIN1 (Nisogi *et al.*, 1992) and yeast MTS3 (Gordon *et al.*, 1996). Amino acid residues identical to those of Cipros1 are indicated by shading. Asterisks represent the residues conserved in all four proteins. Note that these homologues are named Rpn12 according to the proposed nomenclature (Finley *et al.*, 1998).

brary. Screenings for the differentially expressed mRNAs yielded several clones which are predominantly expressed in the eggs and early cleavage stage embryos. One such clone, designated Cipros1, has significant similarity to human 26S proteasome regulatory subunit p31. The deduced amino acid sequence of Cipros1 is 263 amino acids long and its estimated molecular mass is 30.5 kDa. The Cipros1 amino acid sequence is 59.9% identical to that of human p31, while it has less similarity to yeast homologues NIN1 (29.9% identity) and MTS3 (32.2% identity) (Fig. 1). Thus, Cipros1 is ascidian Rpn12 according to the proposed nomenclature of proteasome regulatory subunits.

Using full-length *Cipros1* cDNA as a probe, genomic Southern analysis yielded a single major band and some minor bands (Fig. 2A). Thus, *Cipros1* is assumed to be a single-copy gene in the *C. intestinalis* genome, but there is a possibility that Cipros1-like genes exist in the *Ciona* genome. This is the first report of the molecular cloning of a proteasome regulatory subunit gene from a protochordate.

Expression pattern of *Cipros1* transcript

The expression pattern of *Cipros1* mRNA is intriguing. In Northern blot analysis (Fig. 2B), while the intensity of the bands after the 64-cell stage was constant, the band in the unfertilized egg was at least five-fold more intense than that in the 64-cell stage embryo. Quantitative RT-PCR revealed an expression pattern almost identical to that shown by Northern analysis (data not shown). According to the RT-PCR analysis, during early cleavage stages, *Cipros1* is expressed at a constant level about two-fold higher than the level after the 64-cell stage. Thus, a relatively large amount of *Cipros1* mRNA was maternally expressed and stored in the egg, and the mRNA decreased after fertilization and was expressed at a rather constant level after gastrulation.

The localization pattern of *Cipros1* mRNA was examined by whole-mount *in situ* hybridization (Fig. 3A, B). In unfertilized and fertilized eggs, the *Cipros1* transcript was detected evenly throughout the cytoplasm (data not shown). During the cleavage stage, it was detected in the yolk-free perinuclear

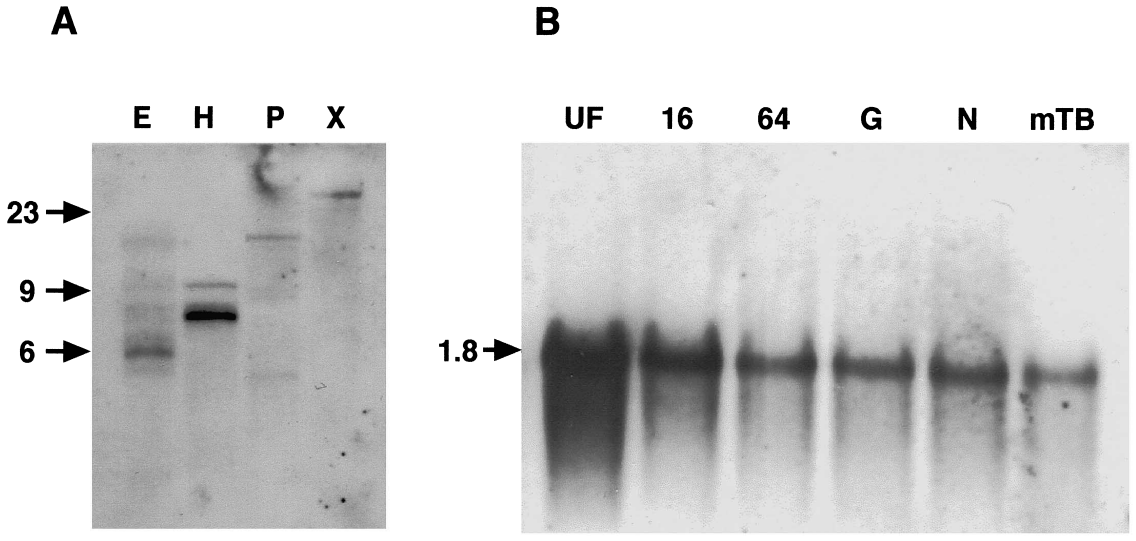

Fig. 2. Southern blot (A) and Northern blot (B) analyses of the *Cipros1* gene. A. *C. intestinalis* genomic DNA was digested with *Eco*RI (E), *Hin*dIII (H), *Pst*I (P) or *Xho*I (X) and hybridized with full-length DIG-labeled *Cipros1* probe. B. Ten micrograms of poly $(A)^+$ RNA prepared from unfertilized eggs (UF), 16-cell stage embryos (16), 64-cell stage embryos (64), gastrulae (G), neurulae (N) and middle tail-bud stage embryos (mTB) were loaded on each lane. Arrows indicate the approximate size in kb.

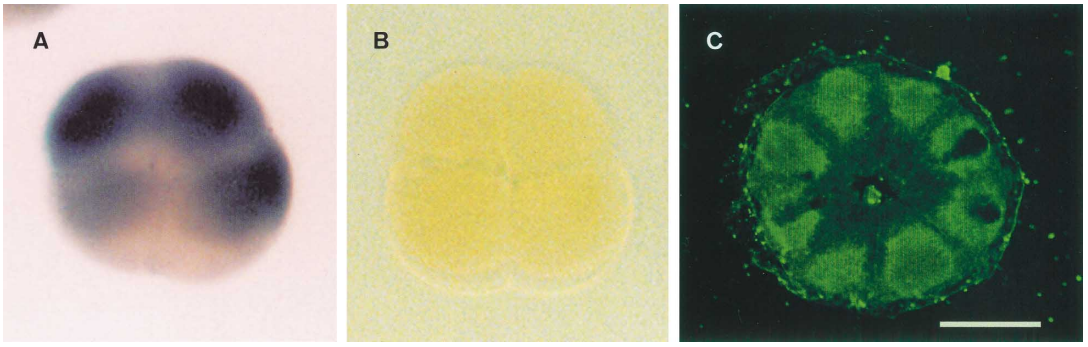

Fig. 3. Spatial patterns of expression of *Cipros1* mRNA and protein. Whole-mount *in situ* hybridization of an 8-cell stage embryo hybridized with antisense (A) and sense (B) DIG-labeled *Cipros1* probe. C. Immunocytochemical staining of a horizontal section of a 16-cell stage embryo with PS1 monoclonal antibody. Scale bar, 50 μm.

cytoplasm of all blastomeres.

Expression pattern of Cipros1 proteins

In order to examine the Cipros1 protein expression, we raised a monoclonal antibody (PS1) against T7-tagged 6 × His-Cipros1 fusion protein. PS1 recognized Cipros1 fusion protein specifically and stained a single band (30 kDa) in the Western blot analysis of the homogenate of *C. intestinalis* gonad (Fig. 4). A constant level of Cipros1 was detected in Western blots of proteins from various developmental stages from fertilized eggs through late tail-bud stage embryos (Fig. 5). However, the Cipros1 band was about twice as intense in unfertilized eggs as in other developmental stages. This is thought to indicate a higher proportion of the transcripts in the

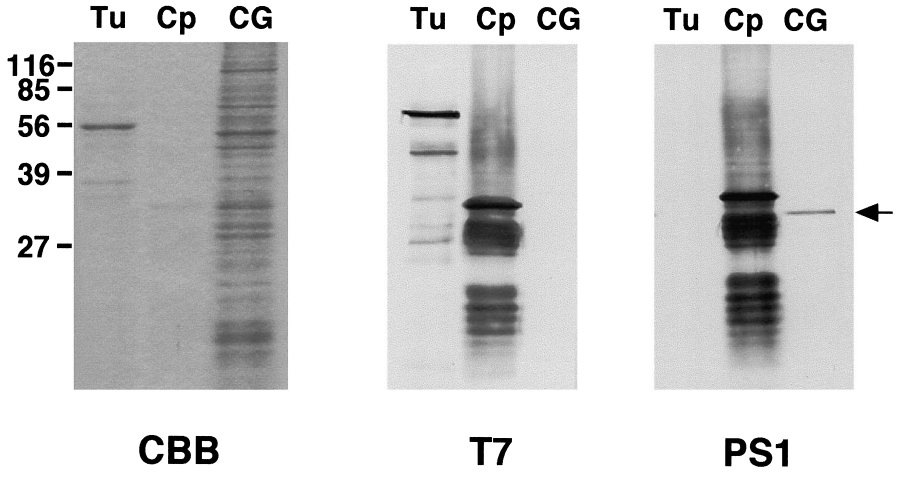

Fig. 4. PS1 monoclonal antibody specifically recognizes Cipros1 fusion protein and *Ciona* endogenous protein. Purified inclusion bodies from two *Esherichia coli* clones which were induced to produce T7-tagged tubulin fusion protein (Tu) and T7-tagged Cipros1 fusion protein (Cp), and total proteins of *C. intestinalis* gonad (CG) were subjected to SDS-PAGE and Western blotted with anti-T7 tag antibody (T7) and PS1 monoclonal antibody (PS1). Total proteins in the gel were stained with Coomassie Brilliant Blue (CBB). Molecular mass is indicated on the left side. PS1 antibody recognized the T7-tagged Cipros1 fusion protein but not the T7-tagged tubulin fusion protein. For all major bands detected by PS1 antibody were also detected by anti-T7 tag antibody, these bands were proteolytic fragments or incomplete forms of the fusion protein. Moreover, PS1 antibody recognizes a single band (30 kDa; arrow) in the *Ciona* gonad proteins.

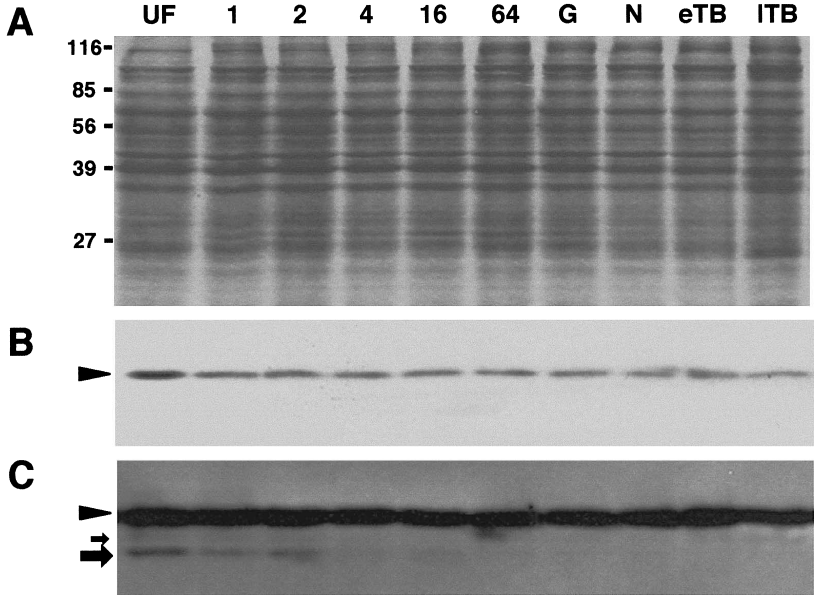

Fig. 5. Temporal expression pattern of Cipros1 protein during early development. Equal amounts of proteins of dechorionated unfertilized eggs (UF), fertilized eggs (1), 2-cell (2), 4-cell (4), 16-cell (16), and 64-cell (64) stage embryos, gastrulae (G), neurulae (N), early tail-bud (eTB) and late tail-bud (lTB) stage embryos were analyzed by SDS-PAGE and stained with CBB (A), or immunoblotted with the PS1 antibody (B, C). The Cipros1 band in the UF was about two-fold more intense than in the other developmental stages (arrowhead in B). C is a longer exposure of B. Smaller minor protein bands (27 and 28 kDa; large and small arrows, respectively) were detected. The 27-kDa protein was expressed specifically during early cleavage stages.

unfertilized eggs. Moreover, longer exposure of the Western blot revealed two other minor bands: one (28 kDa) is very faint and is expressed at constant levels in all stages, and the other (27 kDa) is detected in the unfertilized egg, fertilized egg and 2-cell stage, and much more faintly in the 4- and 16-cell stages. The molecular nature of these minor bands has not yet been determined. As the *Cipros1* mRNA was detected as a single band in the Northern analysis, such protein bands are suggested to be post-translationally modified isoforms of Cipros1.

Sections of *Ciona* embryos were stained with PS1 antibody. In unfertilized eggs, the entire cytoplasm was weakly stained. Throughout the cleavage stage, the yolk-free area of the perinuclear cytoplasm of all blastomeres was stained (Fig. 3C), and throughout early development, the Cipros1 protein showed no obvious localization.

ACKNOWLEDGEMENTS

We thank Dr. H. Yokosawa for his valuable suggestions and his critical reading of the manuscript. We also thank members of the Otsuchi Marine Research Center, Ocean Research Institute of the University of Tokyo, Education and Research Center of Marine Bio-Resources, Faculty of Agriculture of Tohoku University, Ushimado Marine Laboratory of Okayama University and Mukaishima Marine Biological Laboratory of Hiroshima University for facilitating the collection of animals. This work was supported by the "Research for the Future" Program from the Japan Society for the Promotion of Science (96L00404 to T.N.), Grant-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan (10174228 to T.N.) and by a Visiting Researcher grant from the Ocean Research Institute of the University of Tokyo. K.J.T was a doctoral fellow of JSPS with a research grant (8110).

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(Received October 20, 1998 / Accepted November 6, 1998)