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

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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 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...
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'-CGAAACAGCTATGACCATG-3') or P (5'-TGATCGGTAGTCGATAGTG-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.

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-
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 an 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

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**Fig. 2.** Southern blot (A) and Northern blot (B) analyses of the Cipros1 gene. A. C. intestinalis genomic DNA was digested with EcoRI (E), HindIII (H), PstI (P) or Xhol (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.
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 Ciona 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 cytoplasm of all blastomeres.

Western blot analysis of the homogenate of Ciona 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 cytoplasm of all blastomeres.

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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.

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REFERENCES


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