Regulatory mechanisms governing the oocyte-specific synthesis of the karyoskeletal protein NO145

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Summary

Given the prominence and the biological importance of the nucleus it is remarkable how little is still known about structure-forming proteins in the nucleus interior. The karyoskeletal protein NO145 has been identified as a major constituent of a filamentous network surrounding the amplified nucleoli of Xenopus laevis oocytes. We now show that an orthologous protein also occurs in female germ cells of a wide range of other vertebrates, where it forms dot-like structures. Using the Xenopus oocyte system we further report a specific regulatory mechanism responsible for (1) the rapid degradation of the NO145 protein during meiotic maturation, and (2) the cell-type-dependent translation of NO145 mRNA. Microinjection experiments have revealed that NO145 is a target of proteasomes and the use of the rapid amplification of cDNA ends-polyadenylation test (RACE-PAT) has disclosed the existence of NO145 mRNAs differing in their 3′ UTRs. Reporter systems as well as polyribosome profiling experiments have revealed the regulatory importance of the 3′ UTRs, which affect the translational efficiency as well as the stability of the encoded protein. The highly conserved cell-type specificity and the extremely tight temporal regulation of NO145 synthesis suggest an important role of this protein in female meiotic prophase.

Key words: Nucleolus, Maturation, Proteasomes, Karyoskeleton, 3′ UTR

Introduction

Among the morphologically distinct intranuclear structures (Handwerger and Gall, 2006), the nucleolus is the most prominent and functionally best defined nuclear entity, and its molecular composition and functions have been the object of intense investigations. It has been well established for half a century that the nucleolus is the site of ribosomal RNA synthesis, processing and of ribosome maturation, and it was therefore widely regarded as a ‘ribosome producing machinery’ (for reviews, see Hernandez-Verdun, 2006; Raska et al., 2006).

However, recent studies have further indicated that the nucleolus is also involved in other cell functions such as the maturation of various ribonucleoprotein particles (RNPs), the control of the cell cycle and cellular aging processes, and may act like a stress sensor that can, for example lead to the stabilization of the tumor suppressor protein p53 (Gerbi et al., 2003; Mayer and Grummt, 2005; Olson et al., 2002). Moreover, the most recent data from nucleolar mass spectrometry analyses identified ~700 nucleolar proteins, among them many with no obvious relation to ribosome biogenesis (Andersen et al., 2005; Coute et al., 2006; Hinsby et al., 2006). Somewhat surprisingly, mapping the molecular components of nucleoli has not yet revealed insight into molecules that might be involved in the formation or maintenance of the nucleolar structure.

The oocytes of amphibians and certain other eukaryotic organisms contain a special class of multiple nucleoli. For example, the germinal vesicle (GV) of Xenopus oocytes contains up to 1,500 extrachromosomal nucleoli that are assembled on amplified copies of rRNA genes and show a structural organization remarkably similar to that of somatic nucleoli (Gall et al., 2004; Mais and Scheer, 2001). Owing to their abundance and enormous sizes, amplified nucleoli of Xenopus laevis provide an excellent model system for studies of the biochemical composition and structural organization of nucleoli and their dynamic changes during oogenesis and egg formation. In particular, during oocyte maturation the numerous nucleoli disappear in correlation with the breakdown of the nuclear envelope and the disassembly of the nuclear lamina. This characteristic transformation allows the investigation of dramatic alterations in the various nuclear and, in particular, nucleolar structures at the molecular and ultrastructural level.

In addition, a major structural component has been observed in amplified nucleoli of Xenopus laevis oocytes as a cortical network of filaments and knot-like aggregates (Franke et al., 1981; Krohne et al., 1982). More recently, we have succeeded in the identification and molecular characterization of NO145 as the major protein component of this skeletal structure (Kneissel et al., 2001). The cortical meshwork of nucleolar filaments containing NO145 is a very stable, karyoskeletal structure present throughout all stages of oogenesis. However, this protein becomes completely disassembled and dispersed during meiotic maturation, i.e. egg formation. During this period, when all other nucleolar components are distributed throughout the ooplasm, NO145 protein is rapidly and specifically degraded. In order to unravel the biological role of...
NO145 protein, we have examined the mechanisms regulating NO145 during oogenesis, oocyte maturation and the early embryonic development of *Xenopus laevis*. Moreover, we report for the first time on the identification and localization of NO145 orthologous proteins in other vertebrates, mammals included.

**Results**

**Protein NO145 and its mRNA in *Xenopus* oocytes and eggs**

Recently, we reported on the identification of a novel type of karyoskeletal protein termed NO145 in *Xenopus* oocytes as a molecular marker of the cortex of amplified nucleoli characteristic for this cell type (Kneissel et al., 2001). Double-label immunolocalization experiments disclosed that protein NO145 is part of a novel subnuclear structure, i.e. a cortical meshwork representing the outermost portion of the granular component (Fig. 1A). Whereas protein NO145 can be classified as a bona fide karyoskeletal protein, resistant to extraction with high-salt buffers and detergent, it is also highly sensitive to regulated proteolysis. Thus, protein NO145 is present throughout all stages of oogenesis but is rapidly degraded during meiotic maturation, which can be induced in vitro by progesterone treatment. At the time of GV breakdown (GVBD) the protein was no longer detectable, in contrast to the nucleolar protein NO38/B23 which could be found in similar amounts in stage VI oocytes as in eggs, where it appeared in a hyperphosphorylated form (Fig. 1B). Remarkably, northern blot analysis revealed a striking stability of the NO145 mRNA during egg formation (Fig. 1C).

The decrease in NO145 protein during oocyte maturation did not correlate with an increase in NO145 mRNA instability. Our recent studies were aimed at unraveling the mechanism regulating a certain quantity of NO145 during oogenesis, oocyte maturation and early embryonic stages.

**The 3’ UTR of mRNAs encoding protein NO145 determines its translational status**

Using a panel of NO145-specific antibodies, the protein could not be detected in any other cell type beside oocytes, both by western blotting and immunolocalization studies (Kneissel et al., 2001). Therefore, we concluded that the protein represented a specific component of the amplified nucleoli abundant in the amphibian oocyte.

The expression pattern of NO145 mRNA in several major tissue types from *Xenopus laevis* was characterized using reverse transcriptase (RT)-PCR analysis with NO145-specific primers (Fig. 2A, upper panel). The synthesis of NO145 mRNA was restricted to oocytes, as no amplification was seen from sperm, muscle and heart cDNAs. As an internal control for reverse transcription, the various tissue cDNAs were amplified in parallel with primers against the ubiquitous nucleolar protein NO38 (Fig. 2A, lower panel). However, the exclusive amplification of NO145 in the ovary could not rule out the possibility of limited NO145 gene expression in small subsets of cells in other tissues.

Since the poly(A) tail at the 3’ end of most eukaryotic mRNAs plays a critical role in message translation and stability, we also looked for alterations in poly(A) tail length of NO145 mRNA using the RACE-PAT method (Fig. 2B). Interestingly, in all stages of oogenesis two distinct PCR products could be identified which varied slightly in size. By contrast, only the more rapidly migrating band could be seen after in vitro maturation, i.e. in the egg. Western blot analyses revealed a close correlation between the amplification of two PCR products and detection of the encoded protein (Fig. 2C). After cloning and sequencing of the PCR products, the difference became obvious: the larger PCR product, exclusively found in oocytes, represented an extended version of the shorter one detectable in eggs (Fig. 2D). It contained an authentic nuclear polyadenylation signal and a short poly(A) tail. Therefore, we concluded that the absence of the long version of NO145 mRNA was a systemic difference between oocyte and egg.

As NO145 protein was exclusive to immature oocytes, i.e. before GVBD, we hypothesized that this protein might be translated from the long version of the corresponding mRNA. To test this, cellular extracts prepared from oocytes and eggs were fractionated through a 20-60% Nycodenz gradient known to separate free proteins and polyribosomes from mRNPs. Fractions were collected from these gradients and their RNAs were analyzed by the RACE-PAT method. A significant fraction of the NO145 mRNAs from oocytes was associated with ribosomes (fractions 21-23), a finding indicating that NO145 mRNA was translated. By contrast, in the egg, NO145 mRNA was exclusively found in the nonribosomal messenger RNP fraction (fractions 11-15; Fig. 3A). This result suggested that in the absence of the long form, the short form of the NO145 mRNA was stored but in a translationally inactive form. In a control experiment the same fractions were analyzed for the distribution of histone H4 mRNA. This mRNA was recovered in masked mRNPs in the oocytes, but after maturation appeared in a significant portion in association with the polyribosome fraction, in line with the appearance of histone H4 in the egg (Fig. 3B) (Meric et al., 1997).

Obviously, the presence of NO145 protein during oogenesis is tightly regulated at the translational level dependent on the presence of the long version of NO145 mRNA. The
disappearance of the latter perfectly correlates with the specific degradation of the protein.

**NO145 protein is degraded by the proteasome pathway**

To determine the pathway of protein NO145 degradation more precisely, we analyzed the presence and disappearance of the protein upon induction of oocyte maturation. Immunoblot analyses of the various oocyte extracts disclosed the presence of NO145 in untreated stage VI oocytes as well as in oocytes treated with progesterone for 120 minutes. Notably, at the time of GVBD, i.e. the appearance of the white spot, the protein was still detectable, but with significantly slower electrophoretic mobility. Analyses of white-spot oocytes at later times (15, 30, 60 and 120 minutes after GVBD) revealed that NO145 protein levels decreased by 100% between 60 and 120 minutes after GVBD. By contrast, the level of the nucleolar protein NO38 remained almost unchanged during these time intervals. RACE-PAT analyses performed in parallel confirmed the previously observed close correlation between the presence of the long form of NO145 mRNA and the detection of the protein (Fig. 4A).

Before NO145 protein was completely undetectable in mature oocytes and in eggs, it displayed a decreased electrophoretic mobility in early white-spot oocytes compared to immature oocytes. This phenomenon might be due to certain post-translational modifications of the polypeptide such as phosphorylation and ubiquitinylation. First, we tested whether the slow migrating NO145 is phosphorylated and found that on treatment with alkaline phosphatase, NO145 in matured oocytes migrated as fast as that in stage VI oocytes (Fig. 4B).

Second, we examined the role of the proteasome in NO145 degradation, by studying the influence of proteasome inhibitors on the metabolism of endogenous NO145 in vivo. To avoid perturbing early events in maturation that may require proteasome activity, we used the experimental concept...
Oocyte-specific protein NO145 described by Reverte et al. (Reverte et al., 2001), schematically shown in Fig. 4C.

We analyzed the effect of methylated ubiquitin (Met-Ub) on NO145 by injecting this derivative into immature oocytes, followed by induction of maturation and analysis of NO145 by immunoblotting (Fig. 4D). Notably, Met-Ub slowed down the degradation of NO145, allowing the detection of the protein even 120-180 minutes after GVBD (M120 and M180). Moreover, the appearance of a high molecular mass form of NO145 (M30 and M60) additionally indicated that NO145 becomes ubiquitylated during maturation. To obtain further support for the ubiquitin-dependent degradation of NO145, we used the membrane-permeant peptide aldehyde MG132, which has been shown to inhibit the chymotryptic activity of the proteasome (reviewed by Lee and Goldberg, 1998). The injection of MG132 into oocytes at GVBD50 successfully blocked the degradation of NO145 that occurred during the progress of oocyte maturation, whereas injection of 10% DMSO alone did not. A similar biological effect occurred with another, even more specific inhibitor, lactacystin, acting as a pseudosubstrate covalently linked to the active site of the enzyme. Again, injecting this substance prevented degradation of NO145 almost completely (Fig. 4E). Significant alterations of the level of NO145 were apparent when normalized against the amount of the Xenopus helicase Xp54, known to be maintained at a constant level throughout oogenesis and oocyte maturation (Ladomery et al., 1997).

Substrates recognized by the proteasome-ubiquitin system often contain signal sequences that target them for degradation. NO145 contains two putative D-box sequences (RxxLxxxxN) (Glotzer et al., 1991) at aa 266-269 and 601-604, respectively, as well as a potential KEN-box (KEN) (Pfleger and Kirschner, 2000) at aa 347-349. A mutational analysis has been initiated to examine if these sequences might specify NO145 degradation.
All these experiments pointed to a fundamental role of the proteasome in the sharply regulated degradation of NO145 protein during maturation.

Developmental expression of NO145 protein and mRNA
Since one of the NO145 mRNAs, i.e. the short version, was still present in eggs, where no NO145 protein could be identified, we examined at which stage the protein might reappear during embryogenesis. Western blot analysis revealed that no NO145 protein could be identified in early embryonic stages up to the tailbud stage, in contrast to another nucleolar protein, NO66, which was found in all stages in similar amounts (Fig. 5A) (cf. Eilbracht et al., 2004). However, Northern blot analysis revealed that NO145 mRNA was still present, even beyond stage 8 (midblastula transition; MBT), a time when maternally encoded, but translationally inactive, mRNAs are usually degraded (Audic et al., 1997; Voeltz and Steitz, 1998). Remarkably, the NO145 mRNA showed an exceptional stability throughout these stages (Fig. 5B).

RACE-PAT analyses using total RNA from embryonic stages revealed that only the shorter – translationally silenced – version of the NO145 mRNA was present in most stages. Nevertheless, we noted the appearance of the longer mRNA version in stages 9, 11 and 12 exactly upon the onset of zygotic transcription. However, no protein NO145 could be detected which might be caused by the limitation of the immunological assay (Fig. 5C). Given these changes in the abundance of NO145 in oocytes and eggs, as well as during early development, it was tempting to speculate that this protein is only required during oogenesis.

NO145 3’ UTRs affect translational efficiency and stability of the encoded proteins of reporter genes
In many examples, 3’ UTR sequences of mRNAs are known to play a role in regulating their translation (Richter, 1999; Wickens et al., 2000), pointing to the possibility that the 3’ UTRs of the NO145 mRNAs might contain cis-acting elements of a so far unknown identity that function in a similar way. To examine this possibility, the two NO145 3’ UTRs were subcloned downstream of the firefly luciferase coding region (Fig. 6A). mRNA was transcribed in vitro from these constructs, with a poly(A)50 tail added, and was injected into stage VI oocytes together with Renilla luciferase mRNA as an internal control. Four hours later, the oocytes were homogenized and the activities of the firefly and Renilla luciferases were quantitatively measured. Both NO145 3’ UTRs resulted in significant increases of luciferase translation relative to the control, but the long NO145 3’ UTR resulted in significant increases of luciferase translation relative to the control, but the long NO145 3’ UTR was more effective, resulting in an almost twofold increase relative to the short NO145 3’ UTR (Fig. 6B). These variations were obviously due to translational control and did not result from differences in mRNA stability as controlled by recovery and gel electrophoretic analyses of 32P-labeled mRNAs upon microinjection (data not shown).

In a second approach, we fused the two NO145 3’ UTRs to the coding region of the nucleolar protein NO38 carrying a His-tag at its N terminus. The wild-type version of NO38 with its authentic 3’ UTR was used here as a control (Fig. 6C). Equal amounts of each mRNA transcribed in vitro were injected into oocytes, incubated overnight and some oocytes were finally treated with progesterone to induce maturation. Proteins in cellular extracts were separated by SDS-PAGE and analyzed by immunoblotting using the anti-His antibody. All three polypeptides were present in similar amounts in the oocytes (Fig. 6D), although the translation efficiency from the construct carrying the short NO145 3’ UTR was slightly reduced, corresponding to the translational reduction observed in the luciferase assay. Most remarkably, in the egg NO38 protein encoded by the mRNA carrying the short NO145 3’ UTRs (NO38-s) was significantly reduced compared to proteins encoded by NO38wt and NO38-l mRNAs (Fig. 6E). Since all three proteins showed similar turnover rates in oocytes (data not shown), this result indicated to us that the short NO145 3’ UTR directly influenced the stability of the protein during maturation.

The 3’ UTRs of NO145 mRNA contain unidentified regulatory elements affecting both the translational efficiency and the stability of the encoded protein under different biological conditions, here oocyte versus egg.

Identification of mammalian proteins orthologous to Xenopus NO145
During the original characterization of Xenopus NO145 protein, we had noticed a striking homology to SCP2, a rat
synaptopenomal complex (SC) protein of 173 kDa (EMBL accession no. Y08981) (Offenberg et al., 1998). Whereas the N terminus of both proteins contains a domain of 229 aa with a remarkably high sequence homology (43% identity), the overall amino acid sequence identity is only 26% (Kneissel et al., 2001). However, no significant homologies of the *Xenopus* protein with other vertebrate proteins were found. Recently, searches of current databases have disclosed a striking homology between NO145 and human SCP2 (25% aa sequence identity), suggesting that we have indeed cloned the human orthologue of NO145 (Fig. 7B).

Western blot experiments on proteins of various tissue lysates revealed a cell-type-specific expression pattern of the human NO145 protein (Fig. 7C,C′). Antibody hSNO145-5 recognized its antigen exclusively in the lysates derived from ovary but not from testis and intestine (Fig. 7C′, upper panel). This indicated to us that the human orthologue of NO145 shows the same restriction of expression as the *Xenopus* counterpart, i.e. an exclusivity for oocytes. The identity and integrity of the fractions was ascertainment by probing a parallel nitrocellulose filter for the intermediate filament protein vimentin (Fig. 7C′, lower panel).

Next, we examined the intracellular location of hSNO145 by immunofluorescence microscopy. Again, the protein was exclusively detectable in the nuclear compartment of primary oocytes. In contrast to the *Xenopus* protein, however, hSNO145 did not localize to the nucleolus, but accumulated in certain nuclear granules and sizable aggregates (Fig. 7D-D′). All other tissues investigated (e.g. testis, epidermis, liver, scalp and pancreas) and cell culture lines examined did not show any specific reaction.

The observed exclusive expression of hSNO145 in human oocytes will render future experimental work difficult. In order to find a more accessible experimental system, we have tested the antibodies raised against human protein NO145 by immunoblotting on protein lysates from bovine oocytes. Indeed, serum hSNO145-1A showed a strong reaction with a 116 kDa protein in bovine oocyte lysates (Fig. 8A), and localization studies on isolated bovine follicles confirmed that NO145 was exclusive to oogenesis: it was detectable in small oocytes where it localized to nuclear dot-like structures (Fig. 8B,B′), which were not associated with nucleoli (Fig. 8C-C′). The relatively high cytoplasmic background staining might be due to the whole-mount staining procedure. In contrast, MI oocytes and embryos at the 1-cell, 2- to 4-cell, and blastocyst D6 stages did not show any NO145 staining (data not shown).
These results suggest to us that the mechanisms regulating the cell-type-specific synthesis of NO145 protein and its intracellular localization are very similar, if not identical in higher vertebrates and in *Xenopus*. The detection of the protein is restricted to the germinal vesicle of oocytes, which indicates that its function in all these species might be specific to the meiotic prophase of female germ cell formation.

**Discussion**

The first important conclusion from the results of this study is that NO145, originally discovered as a major nucleus-specific protein in the cortices of amplified nucleoli of *Xenopus* oocytes, is a general component of oocytes of many, probably all vertebrates. Together, the cell-type exclusivity and the high amino acid sequence conservation during evolution, from *Xenopus* to man, is indicative of an important functional role of this protein in the meiotic prophase. It was somewhat surprising to find the differences of topology of this protein, from an exclusive integration in the structural arrangements in the nucleolar cortex in amphibian to formations of intranuclear aggregates elsewhere in the nucleus in mammals. Clearly, this polymorphism and also the partial sequence homology to the synaptonemal complex protein, SCP2, have to be taken into consideration in our future studies aiming at an elucidation of the apparently general role of NO145 in female meiosis and oogenesis.

Another remarkable aspect of the synthesis and functionality of NO145 is the extremely sharp regulation of the cell type-specific longevity of the protein as well as of the mRNAs encoding it. The study of this problem has led us to the discovery of an extremely precise regulation of the presence of NO145 both at the protein and at the mRNA level. Our studies in *Xenopus* oogenesis have shown that regulated protein degradation is a significant mechanism in controlling the level of NO145 protein: essentially all of the protein is degraded shortly after GVBD, the first visible sign of the completion of meiosis I. This decrease of NO145 protein concentration is apparently not related to alterations in NO145...
mRNA levels, which essentially remain stable throughout oocyte maturation. In addition, we have found in this study that NO145 degradation in late meiosis I is effected by the proteasome pathway, which is known to be important also for the regulation of many other cellular functions, including cell cycle, growth and polarity (Hegde, 2004; Obin et al., 1999; Ozdamar et al., 2005). The observed phosphorylation occurring immediately upon appearance of the white spot might help to tightly regulate the timing of degradation. In the future it will be interesting to examine whether phosphorylation-dephosphorylation and degradation of NO145 are two coupled events during MI-MII transition. Recently, it has been shown that coordinated degradation of germine-specific proteins is essential for remodeling the oocyte into a totipotent zygote capable of somatic development (DeRenzo and Seydoux, 2004).

Sharp regulation of the life-time of NO145 protein has also been noted in the oogenesis processes of other species. As the protein is absent in mature oocytes and early embryos of *Xenopus laevis*, the human and bovine proteins are also present in early oogenesis and meiosis I, but disappear in later stages. Our immunological studies have disclosed the enrichment of NO145 protein in nuclear granules exclusive to primary and secondary oocytes, whereas later stages were negative for NO145. Similarly, in the bovine system a positive immunological reaction was restricted to small and middle sized oocytes. According to Fair et al. (Fair et al., 1996; Fair et al., 2002) follicles with diameter smaller than 3 mm contain growing oocytes with active RNA transcription. It is these type I and II oocytes that display the NO145-positive granules, whereas type III oocytes, which are characterized by decreasing transcription, only show some weak ‘residual’ reactions with the NO145 antibody. In contrast, NO145 protein becomes undetectable during nuclear compaction in type IV and V oocytes, which are transcriptionally silenced and is also not detectable in mature oocytes and during early embryogenesis. This characteristic behavior with a sharp, early oocyte-specific peak distinguishes NO145 protein from other nuclear proteins (cf. Baran et al., 2004; Fair et al., 2001).

Posttranscriptional control of maternal mRNAs is a principal means of regulating protein synthesis in early developmental stages (Tadros and Lipshitz, 2005). Our observation that the disappearance of NO145 protein during oocyte maturation is not correlated with the loss or complex inactivation of its mRNA prompted us to analyze the NO145 mRNA, in particular its 3’ UTR, in more detail. Most interestingly, we identified two distinct NO145 mRNA populations in oocytes but only one in eggs. These mRNAs differ by only 43 nt in their 3’ UTR lengths, adding a polyadenylation signal and a small poly(A) tail to the shorter version which thus ends with a poly(A) stretch. This relatively unspectacular difference, however, dramatically affects the translational efficiency of the mRNA: the protein is exclusively found in the presence of the long version of the NO145–3’UTR, i.e. in oocytes of stage I–VI as well as in early maturing oocytes (M0–M60), but is not detectable in the presence of only the short NO145 mRNA, i.e. in fully matured oocytes (M120 and eggs) and early embryonic stages. To our knowledge, this kind of regulatory mechanism has not yet been described for any other protein and its mRNA.

In general, changes in polyadenylation can lead to changes in mRNA translation, with increases in poly(A) tail length being associated with translational activation whereas shorter tails result in silencing (Gray and Wickens, 1998). Such changes in the polyadenylation status and, consequently, in translation are also known to be important regulatory principles during male and female gametogenesis (Kashiwabara et al., 2002; Mendez and Richter, 2001). Moreover, it is now widely believed that the length of a poly(A) tail influences the translational efficiency, based on the ‘closed-loop model’ by Munroe and Jacobson (Munroe and Jacobson, 1990). In such a regulatory scenario the shorter version of NO145 mRNA might not allow circularization which would explain the translational repression. On the other hand, translational silencing might be due to the association of the specific mRNA with discrete mRNP complexes. Specifically, it has been shown that Y-box proteins, like the FRGY2 protein, function as translational repressors (Matsumoto and Wolffe, 1998) and that the DEAD-box helicase Xp54 oligomerizes on masked mRNAs and also represses translation (reviewed by Weston and Sommerville, 2006). The observed association of the small NO145 mRNA with polysomes in oocytes (cf. Fig. 3) is somehow confusing. However, at the moment we do not know if indeed both mRNAs become translated. Both may bind to an oocyte-specific factor, which is lacking in the egg, and thereby mediating interaction with ribosomes. Silencing of the short form could be explained by an improper spatial distance between the termination codon and the 3’ end of the mRNA leading to translational repression after initiation (Inada and Aiba, 2005). In addition, specific microRNAs may exist that inhibit actively translating polyribosomes, as recently described (Nottrott et al., 2006).

The majority of maternal mRNAs are stable during oogenesis. Upon oocyte maturation, however, a ‘default
deadenylation’ process occurs, typical of ‘housekeeping’ mRNAs such as actins and ribosomal proteins. This relatively slow event does not depend on the presence of specific cis sequences and, therefore, provides a simple mechanism to inactivate diverse maternal mRNAs simultaneously. This default deadenylation is usually a prerequisite for degradation of certain mRNAs during the blastula stage of embryogenesis (Audic et al., 1997; Duval et al., 1990). Clearly, however, NO145 mRNAs do not fit into this two-step model because the long version is rapidly shortened and then immediately degraded. Obviously, the longer version is not converted into the specific shorter version because the concentration of the latter one remains constant during oogenesis and maturation. Moreover, the short NO145 mRNA is extremely stable and can still be detected at much later stages of embryogenesis, i.e. beyond MBT.

Recently, a number of sequence elements involved in targeting particular mRNAs for deadenylation have been described. These (often AU-rich) elements appear to function as destabilizing sequences by binding distinct proteins and then promoting deadenylation and mRNA turnover (de Moor et al., 2005; Meyer et al., 2004). Whether such a regulatory element is also active in the NO145 3’ UTR remains to be elucidated. Moreover, translational repression and/or activation might be mediated by RNA-protein interactions in the 3’ UTR that do not depend on the polyadenylation status (Robbie et al., 1995). Remarkably, a novel protein termed ePAP [embryonic poly(A) binding protein] has been reported to regulate poly(A) tail length and hence the translatability of mRNAs during Xenopus development (Voeltz et al., 2001). Whether this factor, for which also a special role in the regulation of mRNAs required for gametogenesis has been proposed (Wilkie et al., 2005), might also affect the NO145 mRNA is still unknown.

In conclusion, our studies have shown a very precise spatial and temporal regulation of NO145 synthesis and longevity. Obviously, the translation of NO145 mRNA is controlled at its 3’ UTR by a novel mechanism. Future characterizations of trans-acting factors will help to determine the mechanism and to decide whether this kind of control is specific to protein NO145 or may be more general.

Materials and Methods

**Biological material**

Clawed toads (Xenopus laevis) were purchased from Nasco. Females were induced to ovulate and the subsequent in vitro fertilization was performed as described by Niehrs and De Robertis (Niehrs and De Robertis, 1991). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). In vitro maturation of stage VI oocytes was performed as described previously (Kneissel et al., 2001). Tissue samples from adult X. laevis toads were snap-frozen in isopentane, cooled by liquid nitrogen to about –140°C and stored at –80°C. The human tissues studied were obtained during surgery for medical reasons or during pathological cooling by liquid nitrogen to about –140°C and stored at –80°C. The human tissues

**Preparation of bovine oocytes**

Bovine cumulus-oocyte complexes (COCs) were collected from slaughterhouse ovaries. Follicles of 2-8 mm diameter were either aspirated, using a 19-gauge needle, or recovered by surgery following ovary dissection. Selected COCs were washed twice in M199 maturation medium supplemented with 10% fetal calf serum (FCS; Invitrogen), 10 μg/ml of follicle stimulating hormone (FSH; Rhone Mérieux), 10 μg/ml luteinizing hormone (LH) and 1 μg/ml of estradiol 17β. In vitro maturation of oocytes and in vitro fertilization were done as described previously (Cominelli et al., 2000), except that cumulus cells were removed by vortexing at 18 hours post insemination. Immature and mature oocytes were dechorionized by culture for 3-5 minutes in phosphate buffered saline (PBS) containing 0.5% hyaluronidase (Sigma), then stripped by gentle pipetting before fixation or lysis in denaturing buffer L [50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol]. Lysates were prepared from a group of 40-50 oocytes.

**RNA isolation, in vitro production of mRNAs and [35S]methionine-labeled proteins, and northern blot hybridization**

Total cellular RNAs of X. laevis oocytes, eggs, embryos and tissues were isolated using the TriPure Isolation Reagent (Roche). Capped mRNA was synthesized using the Message Machine T7/T3 kit (Ambion). In vitro synthesis of [35S]methionine-labeled proteins as well as northern blot detection of X. laevis NO145 mRNA were performed as described previously (Kneissel et al., 2001).

**Microinjection of methylated ubiquitin, proteasomal inhibitors and capped mRNAs into Xenopus oocytes**

Upon addition of progesterone, non-white spot oocytes were collected when 50% of them had undergone germlinal vesicle breakdown (GVBD) and they were injected with 50 nl of either 0.5 mM lactacystin (Merck), 1 mM carboxbenzoxyl-leucinyl-leucyl-leucinal (MG132; Merck), 116 μM methylated ubiquitin (Met an Eozki restriction site. For the amplification of the long NO145-3’ UTR, the oocytes showed the white spot (GVBD);105, five oocytes per time point were collected, homogenized and prepared for SDS-PAGE.

Stage VI oocytes were injected with 50 nl of an appropriate dilution of in-vitro-transcribed and capped mRNAs (~60 ng). After 24 hours, oocytes were separated and groups of three oocytes per mRNA were subclassed and analyzed by SDS-PAGE. Where indicated, the lysates were treated with 0.6-6 U/μl calf intestine alkaline phosphatase (Promega) at 37°C for 30 minutes.

**Race-PAT analysis and Nycoenz density gradient fractionation of ribonucleoprotein particles (RNP)s from X. laevis oocytes and egg**

The polyadenylation test on total RNA isolated from Xenopus oocytes, eggs, embryos, and different tissues, respectively, was performed according to the method of Sallés et al. (Sallés et al., 1999) using the gene-specific primers 5’-GACCTCCTAGGCTGGGTCGCT-3’ (NO145) and 5’-CCGGATCTAGACCTTGGATCTTG-3’ (NOS8), respectively.

Extracts from 100 oocytes or eggs were fractionated on a Nycoenz density gradient as described by Tafuri and Wolffe (Tafuri and Wolffe, 1993). Total RNA was isolated from the resulting fractions and further analyzed using the RACE-PAT method.

**Gel electrophoresis and immunoblotting**

Protein fractions were analyzed by SDS-PAGE according to the method of Thomas and Kornberg (Thomas and Kornberg, 1975), and immunoblot analysis was performed as described by Kneissel et al. (Kneissel et al., 2001).

**Tissue protein lysates of human origin were obtained from Imgenex (San Diego, CA).**

**Plasmid constructs**

To generate the various His-NOS3-3’-UTR constructs, the full-length NOS3 cDNA was amplified from plasmid pBT-NOS3 (Schmidt-Zachmann et al., 1987), using the primers 5’-CCGGATCTAGACCTTGGATCTTG-3’, which incorporates a BamHI restriction site. For the amplification of the long NO145-3’ UTR, the antisense primer 5’-GACCTCCTAGGCTGGGTCGCT-3’, which incorporates an EcoRI site at the 3’ end of the product. The short form of the NO145-3’ UTR was amplified from cDNA obtained from X. laevis oocytes using the primers 5’-GACCTCCTAGGCTGGGTCGCT-3’, introducing the same SacI and EcoRI restriction sites, was used. After restriction digest, the NO38 coding sequence was combined with either the short or the long version of the NO145-3’ UTR and subcloned into a modified version of the pET-15 vector (kindly provided by R. A. Kammerer, University of Manchester, UK) previously cut with BamHII and EcoRI, resulting in constructs pHis-NOS3+8 and pHis-NOS3-8. The plasmid pHis-NOS3+8 was generated from wild-type cDNA subcloned into the pET-15b vector using BamHII and EcoRI restriction sites.

The firefly luciferase reporter constructs were generated by amplification of the short NO145-3’ UTR sequence using the primers 5’-GAGCTTCAAGCCACACTGAT-3’, introducing a SruI site and 5’-GACCTCCTAGGCTGGGTCGCT-3’, which incorporates an EcoRI site, was used. After restriction digest, the NO38 coding sequence was combined with either the short or the long version of the NO145-3’ UTR and subcloned into a modified version of the pET-15 vector (kindly provided by R. A. Kammerer, University of Manchester, UK) previously cut with BamHII and EcoRI, resulting in constructs pHis-NOS3+8 and pHis-NOS3-8. The plasmid pHis-NOS3+8 was treated by restriction enzyme SauI and EcoRI to generate the promoterless vector pLuc-MCS (kindly provided by Nancy Standart, University of Cambridge, UK) downstream of the luciferase coding region. The firefly luciferase vector pLuc-MCS was used as a control. For capped mRNA synthesis the plasmids were
linearized with PvuII to give (A30) transcripts. The Renilla luciferase vector pRL-TK (Promega) was linearized with XhoI prior to the synthesis of capped RNA.

**Luciferase activity assay**

50 nl of a mixture containing the firefly luciferase and the Renilla luciferase capped mRNAs were injected into stage VI oocytes with six-fold excess of firefly luciferase over Renilla luciferase mRNA. Oocytes were incubated at 18°C for 4 hours, groups of four oocytes were each pooled and homogenized in 150 μl Passive Lysis buffer (Promega). The homogenate was centrifuged for 5 minutes at 16,000 g and 10 μl of the cleared lysate were assayed for firefly and Renilla luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) in a GENEios luminometer (Tecan, Crailsheim, Germany). Each experiment was repeated several times with oocytes from different frogs.

**Antibodies**

Guinea pig antibodies NO145-I against Xenopus NO145, and NO66-2 against the nucleolar protein NO38 have been described before (Eilbracht et al., 2004; Kneissel et al., 2001). The Xenopus laevis helicase p54 was detected with the rabbit serum anti-p54 (Ladomery et al., 1997), kindly provided by John Sommerville (University of St Andrews, UK), and wild-type nucleolar protein NO38/B23 was either recognized by the mAb No-185 (Schmidt-Zachmann et al., 1987) or by polyclonal antibodies X8 directed against the peptide IAPDASKVPRK (aa 144-155 of Xenopus protein NO38). The His6-tagged version of protein NO38 was recognized by mAb o-His-Tag (Merck). Secondary antibodies used for immunoblotting were horseradish peroxidase-conjugated antibodies (Dianova, Hamburg, Germany).

**Antigen retrieval and immunofluorescence microscopy**

Sections of formaldehyde-fixed and paraffin-embedded human tissues were deparaffinized and treated for antigen retrieval in a microwave oven (Milestone, Bergamo, Italy) for 10 minutes with 1 ml of a mixture containing the firefly luciferase and the Renilla luciferase vector pRL-TK (Promega) was linearized with XbaI (Promega) and 10 μl of the cleared lysate were assayed for firefly and Renilla luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) in a GENEios luminometer (Tecan, Crailsheim, Germany). Each experiment was repeated several times with oocytes from different frogs.

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