Nucleoskeleton and nucleo-cytoplasmic transport in oocytes and early development of *Xenopus laevis*

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ABSTRACT We use amphibian oocytes and eggs as favorite biological systems to study various cell biological phenomena. We have analyzed the role of the zinc finger protein TFIIIA and ribosomal protein L5 in nucleo-cytoplasmic transfer of 5S ribosomal RNA and report on the structural requirements of the 5S RNA for the interaction with TFIIIA. Furthermore, we have used the oocyte/egg system to analyze the kinetics of the posttranslational isoprenylation of oocyte nuclear lamin B3 and its fate during egg maturation. We demonstrate, that isoprenylation of newly synthesized lamins takes place in the oocyte cytoplasm before uptake into the nucleus and show, that the isoprene modifications alone are not sufficient to maintain stable association of lamins with nuclear envelope derived membranes in eggs. Finally, initial results of the identification of cis-acting sequence elements, involved in translational repression of lamin mRNAs in oocytes, are reported.

KEY WORDS: *Xenopus, nucleo-cytoplasmic transport, TFIIIA, lamins, translational control*

Introduction

Amphibian oocytes and embryos have been favorite biological systems for embryologists as well as cell biologists. Amphibian development starts from a comparatively large egg. After fertilization, the egg cleaves rapidly to form a blastula of about 4000 cells within a few hours without a significant expression of the embryonic genes. This developmental strategy requires special mechanisms affecting the processes of DNA replication, chromatin assembly and nuclear envelope formation, transcription, RNA processing and storage, translation as well as protein compartmentation. The materials required for these processes are accumulated during oocyte growth which takes several months. A fully grown *Xenopus* oocyte has a diameter of about 1.2-1.4 mm. The large cell size facilitates micromanipulations like microinjection, manual enucleation and the separation of the nuclear envelopes from the nuclear content of oocyte nuclei (germinal vesicle). Since the fractionation can be done with unsurpassed speed and precision *Xenopus* oocytes have become a classical system to study nucleo-cytoplasmic transport of macromolecules.

Assembly and nucleo-cytoplasmic transport of small RNPs

Pioneering experiments performed by Eddy De Robertis and his colleagues have revealed that the segregation of small RNA molecules between nucleus and cytoplasm is not coupled to transcription, indicating that signals on the RNA molecules themselves and/or associated proteins are primarily important for the regulation of intracellular migration in *Xenopus* oocytes (De Robertis *et al.*, 1982). We have analyzed nucleo-cytoplasmic transfer of 5S ribosomal RNA. In the course of oogenesis, the synthesis of large amounts of 5S ribosomal RNA precedes the synthesis of stoichiometric amounts of other ribosomal components and, hence, ribosomal assembly. Excess 5S RNA is stored in the cytoplasm as a 7S RNP, one molecule 5S RNA associated with one molecule TFIIIA. TFIIIA is a zinc finger protein with a dual function in nucleic acid binding; as a sequence specific DNA binding protein it serves as a 5S gene specific transcription factor, interacting with the internal control region of the 5S RNA gene (Picard and Wegnez, 1979; Pelham and Brown, 1980; Honda and Roeder, 1980). In *Xenopus* oocytes, the specific complex with 5S RNA, the 7S RNP, forms in the nucleus and is then transferred to the cytoplasm for storage. 5S RNA export from the nucleus can also take place in the context of a different small RNP, where the 5S RNA molecule is bound by ribosomal protein L5 (Guddat *et al.*, 1990). This complex has been coined 5S RNP (Steitz *et al.*, 1988). RNA mutagenesis has revealed that 7S RNP and 5S RNP represent alternative pathways for 5S RNA export; furthermore, binding of either of these two proteins is required for 5S RNA transfer to the cytoplasm, since mutant RNA that cannot complex with TFIIIA or L5 is retained in the nucleus (Guddat *et al.*, 1990).

Abbreviations used in this paper: TFIIIA, transcription factor IIIA; L5, ribosomal protein L5; RNP, ribonucleoprotein particle; 3'UTR, 3' untranslated region.

More recently, we were able to demonstrate that the 5S RNA complex with L5, the 5S RNP, is able to shuttle between nucleus and cytoplasm, since it will also travel in reverse direction, from the cytoplasm to nucleus. As for the export, nuclear transfer of 5S RNA depends on protein binding. In contrast to the 5S RNP, the 5S RNA complex with TFIIIA (the 7S RNP) is retained in the cytoplasm (F. Rudt and T. Pieler, in preparation). These observations demonstrate that binding of specific proteins to 5S RNA provides at least part of the signalling information which targets 5S RNA to either cytoplasm (for storage) or nucleus (for ribosomal assembly). We cannot exclude the possibility that other proteins, which we have failed to identify so far, will also play a role in 5S RNA containing RNP trafficking. Competition of tRNA and 5S RNA transport by microinjection of various RNA molecules into the cytoplasm of *Xenopus* oocytes does indeed suggest the existence of common as well as specific RNP transport factors (F. Rudt and T. Pieler, in preparation).

Our simple working hypothesis on nucleo-cytoplasmic transport of 5S ribosomal RNA is illustrated in Figure 1. It is meant to suggest that 5S RNA exchange from 7S RNP into 5S RNP (upon degradation of TFIIIA and/or biosynthesis of excess L5) offers a (hypothetical) mechanism for 5S RNA stored in form of cytoplasmic 7S RNPs to be recruited for ribosome assembly via the 5S RNP shuttling pathway.

Structural requirements for the interaction of 5S RNA with TFIIIA

TFIIIA carries 9 zinc finger modules of the C_2H_2 type in tandem repeat. Of these, the central three units (4 to 6) are sufficient for specific recognition of 5S ribosomal RNA; the amino terminal zinc finger triplet (units 1-3) is sufficient for DNA recognition (Theunissen *et al.*, 1992; Clemens *et al.*, 1993). The RNA binding zinc finger triplet (4 to 6) exhibits several unusual structural characteristics not found in the majority of the many other zinc finger proteins that have been described. The linker region between the invariant zinc coordinating histidines and cysteines of the adjacent zinc fingers 5 and 6 is shortened (4 instead of 7 amino acids) and the finger loop 6 is enriched in aromatic residues, which might be important for RNA binding. In fact, based on these primary sequence characteristics, the tertiary structure of finger 6, which appears to be most important for RNA recognition, must be significantly different from the consensus structure of other DNA binding zinc finger modules which have been solved by 2D-NMR or x-ray structural analysis. Zinc finger DNA recognition is primarily achieved via base specific contacts forming in the major groove of the DNA double helix. The portion of 5S RNA recognized by TFIIIA is a complex arrangement of three helical elements and two internal loops (Fig. 2). In contrast to DNA recognition, RNA binding is not likely to involve primarily base specific contacts; it is the complex 5S RNA secondary/tertiary structure, rather than its primary sequence, which is the major determinant in specific protein recognition (Pieler *et al.*, 1984; You *et al.*, 1991; Theunissen *et al.*, 1992). Multiple contacts with the RNA phosphate backbone are important for complex formation (O. Theunissen and T. Pieler, in preparation).

Although footprinting experiments have revealed extensive protection on the 5S RNA surface, RNA mutagenesis identifies the central core of 5S RNA to be essential and sufficient for
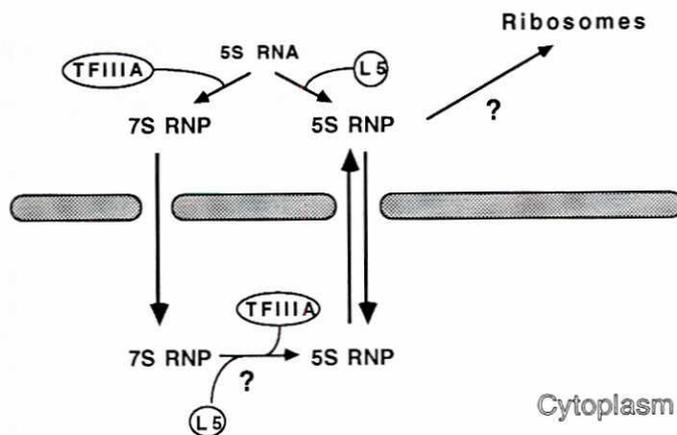

Fig. 1. Working hypothesis on the nucleo-cytoplasmic transport of 5S ribosomal RNA in *Xenopus* oocytes.

TFIIIA binding (Fig. 2). Thus, in summary, RNA and DNA recognition by zinc fingers appear to operate via distinct mechanisms. Base specific contacts govern DNA binding, whereas RNA secondary/tertiary structure is the primary driving force in RNA binding. Elucidation of the precise protein/RNA contacts forming will have to await the exact structural solution of a representative zinc finger/RNA model system, such as the TFIIIA/5S RNA complex.

Analysis of nuclear lamins

Nuclear lamins are the major structural components of the nuclear lamina, the most distinct element of the nucleoskeleton. Lamins are members of the intermediate filament protein multigene family (Aebi *et al.*, 1986; Fisher *et al.*, 1986; McKeon *et al.*, 1986). Lamin filaments line the nucleoplasmic surface of the inner nuclear membrane. They are thought to be involved in nuclear envelope integrity, the anchorage of nuclear pore complexes, and the three-dimensional organization of the interphase chromatin of somatic cells (for review see Gerace and Burke, 1988; Nigg, 1989). Our interest to study lamins in amphibian oogenesis and early embryogenesis is several fold. We have previously shown that the individual types of lamins are differentially expressed in early *Xenopus* development and that the changes in the nuclear lamina composition correlate with major transitions in nuclear function. Moreover, *de novo* synthesis of lamin polypeptides is entirely dependent on the translational activation of maternal lamin mRNAs in this early phase of development (Stick and Hausen, 1985). The mechanisms that underlie this type of control are currently under study in our laboratory.

Analysis of posttranslational modifications and membrane targeting of oocyte nuclear lamin B3

Xenopus oocytes express lamin B3 as their major lamin constituent (Krohne *et al.*, 1981; Stick and Krohne, 1982; Lourim
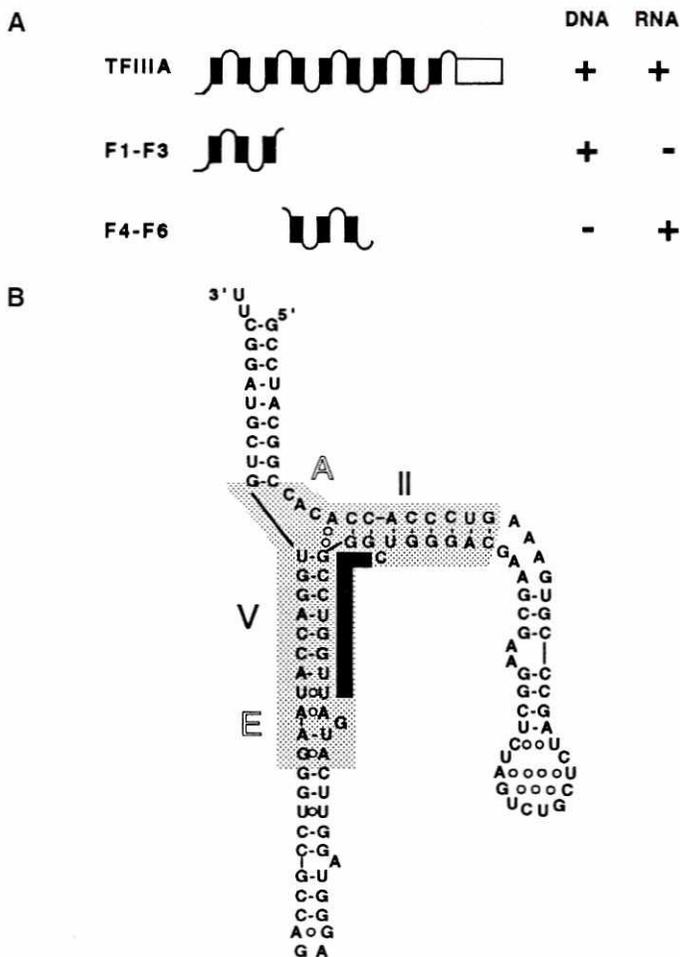
A

	DNA	RNA
TFIIIA	+	+
F1-F3	+	−
F4-F6	−	+

B

Fig. 2. Interaction of 5S RNA with TFIIIA. (A) *Transcription factor IIIA (TFIIIA) contains nine zinc finger modules in tandem array. Zinc finger units 1 to 3 are sufficient for sequence specific DNA binding, finger units 4-6 are sufficient for specific RNA recognition.* **(B)** *Primary/secondary structure of Xenopus laevis somatic 5S ribosomal RNA. Stippling indicates structural elements, which are involved in TFIIIA binding according to RNA mutational analysis. The solid bar indicates phosphate residues found to be important for TFIIIA binding according to modification - interference experiments.*

and Krohne, 1993). The maternal pool of lamin B3 accumulated in the form of the germinal vesicle lamina is subsequently used in the formation of pronuclei and cleavage nuclei. In contrast to mammalian and chicken somatic B-type lamins, which remain associated with remnants of nuclear envelope membranes after mitotic depolymerization, *Xenopus* lamin B3 becomes soluble during nuclear envelope breakdown in meiotic metaphase in the course of egg maturation (Benavente *et al.,* 1985; Stick and Hausen, 1985). This observation that had been made quite some time ago has attracted cell biologists attention for two reasons. First, the soluble lamin can be removed from egg extracts by immunodepletion without affecting other components in the extract. Such lamin depleted egg extracts can be used in *in vitro* nuclear assembly experiments to test how lamins participate in nuclear formation and the organization of DNA replicons. This experimental route has been followed by several groups includ-

ing our own (Newport *et al.,* 1990; Meier *et al.,* 1991; Jenkins *et al.,* 1993). The results indicate that, while lamin B3 is not required for either chromatin decondensation or nuclear membrane assembly, it is essential for initiating DNA replication and may be involved in targeting enzymes to the sites of DNA synthesis.

Second, the dissociation of lamin B3 from nuclear envelope membranes during meiotic nuclear disassembly poses the question of how membrane association of lamins is brought about and how this association is regulated during the cell cycle. Several lines of evidence show that two topogenic sequences are necessary to target newly synthesized lamins to the inner nuclear membrane, a nuclear localization signal (Loewinger *et al.,* 1988) and the carboxyl terminal CaaX tetrapeptide (C= cysteine, a= generally aliphatic) (for review see Nigg *et al.,* 1992). The CaaX motif is the substrate for a series of posttranslational modifications including isoprenylation of the cysteine, proteolytic removal of the last three amino acids, followed by carboxyl methylation of the resulting COOH-terminal cysteine residue. The CaaX-dependent modifications result in an increased hydrophobicity of the carboxyl terminus and may explain the affinity of prenylated proteins for membranes. The group of prenylated proteins is quite heterogeneous, it includes Ras proteins and many of the other small G-proteins, the heterotrimeric large G-proteins, the retinal cGMP phosphodiesterases, most nuclear lamins, and several fungal mating pheromones (for review see Clarke, 1992; Schafer and Rine, 1992). Isoprenylation has also taken to explain the different fates of A- and B-type lamins during cell division (Kitten and Nigg, 1991). A-type lamins, which loose their isoprenyl moiety shortly after incorporation into the lamina structure, become freely soluble upon nuclear envelope breakdown. Somatic B-type lamins, in contrast, are permanently isoprenylated and, although depolymerized during mitosis, remain associated with remnants of nuclear envelope membranes (Gerace and Blobel, 1980; Stick *et al.,* 1988). We have analyzed the posttranslational modifications of *Xenopus* lamin B3 in oocytes (interphase) and in eggs (meiotic metaphase) and we have compared the subcellular distribution of B3 in these stages with that in transfected mouse cell lines expressing *Xenopus* lamin B3. In agreement with previous studies we have shown that isoprenylation of lamin B3 is essential for its targeting to the inner nuclear membrane and the stable integration into the lamina structure. We also found that lamin B3, similar to somatic B-type lamins, is permanently isoprenylated in oocytes and eggs. Moreover, in transfected somatic mouse cells a significant portion of B3 remains associated with membranes during mitosis (Firmbach-Kraft and Stick, 1993). From these results it has been concluded that the CaaX motif-mediated modifications alone, although necessary for membrane targeting, are not sufficient for a stable association of lamins with membranes.

Oocytes have also been used to answer the question when after *de novo* synthesis lamin B3 is isoprenylated relative to its uptake into the nucleus. Previous attempts to answer this question had led to conflicting conclusions (Kitten and Nigg, 1991; Lutz *et al.,* 1992; Sinensky *et al.,* 1994). For this analysis we have compared the kinetics of isoprenylation and nuclear uptake of newly synthesized lamin B3 by pulse chase experiments. The results showed conclusively that lamin B3 is isoprenylated in the

oocyte cytosol before uptake into the nucleus (Firmbach-Kraft and Stick, submitted). This observation raises the question whether newly synthesized lamins are freely soluble in the cytosol or whether they become transiently associated with cytoplasmic membranes on their route to the nuclear pores. In the latter case nuclear uptake must be dominant over association of isoprenylated lamins with cytoplasmic membranes. Taken together, accumulation of unisoprenylated lamins within the nucleus, as it has been described in the analysis of the processing pathway of lamin A in cell culture cells (Lutz *et al.,* 1992), represents a default pathway in isoprene starved cells as shown by inhibitor experiments with *Xenopus* oocytes.

For other isoprenylated, cytoplasmic, proteins it has been shown that isoprenylation is not sufficient to confer stable membrane attachment and that other factors must be involved in a tight membrane binding of isoprenylated proteins (Hancock *et al.,* 1991; Butrynsky *et al.,* 1992). In mammals and birds several integral membrane proteins of the inner nuclear membrane have been characterized that interact with lamins (Worman *et al.,* 1988; Bailer *et al.,* 1991; Simos and Georgatos, 1992; Foisner and Gerace, 1993; Meier and Georgatos, 1994) These are possible candidates to mediate the specific binding of isoprenylated lamins to the inner nuclear membrane. One of these contains several membrane spanning domains of unknown function (Worman *et al.,* 1990). It has been speculated that these hydrophobic domains might interact with the isoprenyl groups of lamins. Different modifications of these receptors might be involved in the modulation of the receptor-lamin interaction during mitotic cell division and meiotic egg maturation.

One of our ongoing projects is the characterization of homologue receptor(s) in the *Xenopus* oocyte/egg system and the analysis of its interaction with lamins during oogenesis and egg maturation.

Translational control of maternal lamin mRNAs

Early development in many organisms including amphibians is directed by maternally inherited mRNAs and proteins (for review see Davidson, 1986). These materials are synthesized during the period of oogenesis. Only a subset of the total diverse messenger population is found on polysomes. More than 90%, by mass, of the polyadenylated RNA (poly(A)+ RNA) in fully grown *Xenopus* oocytes is stored as nontranslating mRNPs. Egg maturation and fertilization are two major transitions at which many of the stored mRNAs are recruited onto polysomes (for review see Richter, 1987). At the same developmental stages changes in polyadenylation status of many mRNAs are observed (Jackson and Standard, 1990).

Although many mRNAs follow the general mobilization during egg maturation and fertilization there are a number of stored mRNAs which exhibit a unique temporal behavior and closer inspection of individual mRNAs suggests that the expression of each mRNA species might be under some specific control. Most interestingly, several mRNAs become activated only later during the blastula stage or during gastrulation, examples for such temporal control in *Xenopus* are the mRNAs encoding histon H1 (Flynn and Woodland, 1980), fibronectin (Lee *et al.,* 1984), c-myc (Godeau *et al.,* 1986; King *et al.,* 1986; Taylor *et al.,* 1986), and several members of the nuclear lamin protein family (Stick
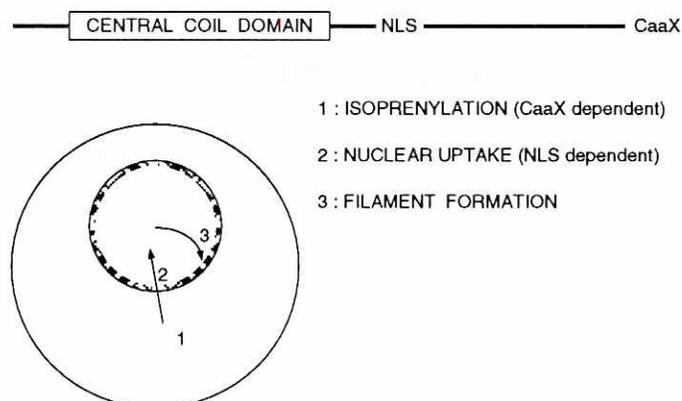

Fig. 3. Steps in nuclear lamin targeting. *Schematic view of the primary structure of nuclear lamins (top). A central coil domain is flanked by non-helical head and tail domains. In the tail domain two topogenic sequences are located: the C-terminal target sequence for posttranslational isoprenylation and proteolytic processing (CaaX) and the nuclear localization signal (NLS). Steps in posttranslational processing and targeting of lamins as revealed by kinetic studies in Xenopus oocytes (bottom). Newly synthesized lamins are isoprenylated in the cytoplasm (1), transported into the nucleus (2), and targeted to the nuclear envelope membrane where filament formation occurs (3).*

and Hausen, 1985). The expression pattern of some of the latter is quite complex. Translation of lamin B1 from maternal mRNA starts with the onset of mid blastula transition (MBT), that of the B2 mRNA during gastrulation. Lamin B3 is translated in oogenesis while during the early cleavage stages, before MBT, B3 is down regulated. Its rate of synthesis increases with the onset of MBT, parallel to the activation of the maternal transcripts of lamin B1. Experiments in which synthesis of mRNA was blocked by administration of α-amanitin demonstrated that the activation of maternal lamin mRNAs during this early phase of development is independent of the onset of embryonic transcription that occurs during MBT.

The proposal that RNA binding proteins can execute regulatory function in maternal mRNA expression is long-standing (Spirin, 1966). For stored maternal mRNA in oocytes and embryos of amphibians and marine invertebrates it has been shown that they exist as "masked" mRNAs, mRNA-protein complexes in which proteins are thought to act as repressors of translation (Rosenthal *et al.,* 1983; Richter and Smith, 1984). In fact, all three lamin polypeptides are efficiently synthesized *in vitro* from protein-free preparations of total oocyte RNA (Stick, 1988). We are currently analyzing the mechanisms that are involved in the translational repression of lamin mRNAs in *Xenopus* oocytes. In a first approach we have concentrated on the identification of cis-acting sequence elements of lamin mRNAs as possible binding sites for repressor proteins. Preliminary results show that sequences of the 3' untranslated region (3'UTR) of the lamin B1 mRNA are involved in translational repression in oocytes. Synthetic RNA encoding lamin B1 that contains the complete 3'UTR is translationally repressed when injected into oocytes while truncated versions of this RNA that lack parts or the entire 3'UTR are efficiently translated. The stability of the injected RNA is not affected by the presence or absence of the 3'UTR. These RNA injection experiments also

indicate that the repressor molecules must be in excess over the endogenous mRNA since the amount of injected synthetic RNA that is repressed by far exceeds that of the endogenous mRNA.

A first goal of our analysis is the characterization of sequence elements that mediate the specific temporal control of lamin mRNAs in oocytes and early embryos. These sequence elements will then be used to characterize the corresponding RNA binding protein(s) as a necessary step to learn more about how the repression of the maternal mRNAs in the oocyte is brought about and how derepression later in the embryo is regulated.

These sequence elements might also be used to program the temporal expression of other proteins in early embryonic development to get insight into their function by their ectopic (parachron) expression.

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