

# Nucleolin from *Xenopus laevis*: cDNA cloning and expression during development

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**Nucleolin is a key nucleolar protein in higher eukaryotic cells and is involved directly in ribosome biogenesis. Using an antiserum raised against hamster nucleolin, the homologous protein was detected in nucleoli of *Xenopus laevis* hepatocytes as well as in the amplified nucleoli of oocytes. A cDNA encoding *Xenopus* nucleolin has been isolated and sequenced. The deduced protein sequence reveals similar domains in *Xenopus* and in mammals, but they have undergone separate evolutions. In particular, each of the four RNA-binding domains has evolved differently—the carboxy-proximal domain is twice as conserved (87%) as the amino-proximal domain (42%). These data shed some light on the possible roles of each domain. The expression of nucleolin has been followed throughout oogenesis and embryogenesis. The appearance of nucleolin during early development precedes the transcription of rDNA and the synthesis of ribosomal proteins. The maximal accumulation of nucleolin at gastrulation coincides with nucleolar reformation. Furthermore, when ribosomal synthesis is activated during oogenesis and embryogenesis, peptides immunorelated to nucleolin appear and accumulate. The results suggest that nucleolin plays a role not only in ribosome assembly but also in nucleologogenesis.**

[*Key Words*: Ribosome biogenesis; nucleologogenesis; nucleolar proteins; RNA-binding domain; oogenesis; embryogenesis]

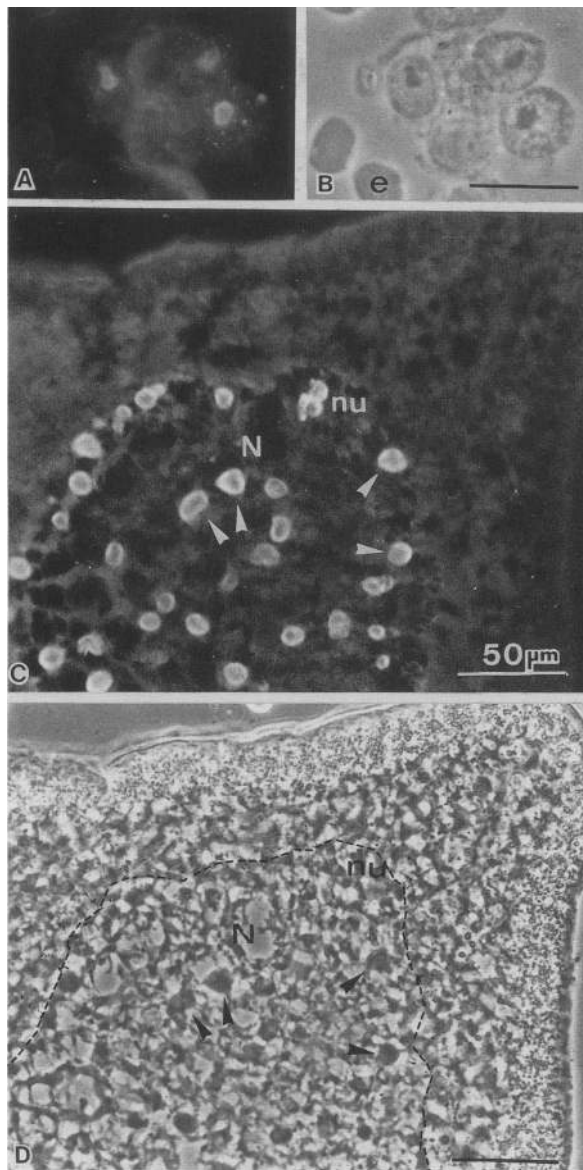
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Ribosome biosynthesis, whose main steps occur in the nucleolus of higher eukaryotic cells, requires the coordinate expression of >100 genes. Not only is the transcription of these genes carried out by three different RNA polymerases, but different post-transcriptional events are also involved. Some RNAs undergo a maturation process within the nucleolus, while other transcripts need to be spliced, exported to the cytoplasm, and translated into proteins which are then transported to the nucleolus. There are many different levels at which controls could be exerted (for review, see Hadjiolov 1985). Until now, attempts to find a common regulatory trigger that could explain the coordinate expression of these genes have been unsuccessful.

Nucleolin is a major nucleolar protein that is ubiquitous to higher vertebrates (Orrick et al. 1973; Pfeifle and Anderer 1983; Sapp et al. 1986). The amount of nucleolin in the cell is correlated directly with nucleolar transcriptional activity (Escande-Géraud et al. 1985; Bouche et al. 1987). It has been shown to be associated with pre-rRNA (Bugler et al. 1987), chromatin (Olson and Thompson 1983; Erard et al. 1988), preribosomes (Bugler et al. 1982; Herrera and Olson 1986), and the nucleolar matrix (Caizergues-Ferrer et al. 1984) and could play a role in the organization of nucleolar substructure.

The complete sequence of the Chinese hamster protein has been established (Lapeyre et al. 1987), and more recently, the gene encoding nucleolin was isolated and characterized in rodents (Bourbon et al. 1988a). The sequence reveals several interesting features including a striking array of four RNA-binding domains.

We were interested in studying the pattern of expression of nucleolin to get an insight on the regulation of ribosome biogenesis. For such an investigation, *Xenopus laevis* is a useful model system due to the existence of two developmental situations in which ribosome synthesis represents a major effort of the cell: (1) following rDNA amplification in oogenesis, when a huge amount of ribosomes is produced from the amplified nucleoli and stored for use during early development; and (2) in embryogenesis, when the maternal stock of ribosomes is used up and the embryo begins to synthesize new ones. The relative timing of expression for rRNA, 5S RNA, and ribosomal proteins is well documented in *Xenopus* development (for review, see Davidson 1986). The reformation of nucleoli beginning in gastrula is correlated with a weak revival of rDNA transcription (Busby and Reeder 1982) and a slow recovery of ribosomal protein synthesis (Pierandrei-Amaldi et al. 1982). Because in mammalian cells, at the end of mitosis, nucleolin is



**Figure 1.** Nucleolin localization. Indirect immunofluorescence microscopy on isolated liver nuclei from *Xenopus* using the purified antiserum raised against CHO nucleolin (A) and corresponding phase contrast (B). Nucleoli are stained positively. Erythrocytes are not stained (e). Frozen section of *Xenopus* ovary (C) showing part of an oocyte nucleus (N) with numerous nucleoli after incubation with anti-nucleolin antibody. The amplified nucleoli (nu) at this stage (st. IV) are strongly fluorescent. (D) Phase contrast: the nucleus is circled. Bar, 50  $\mu$ m.

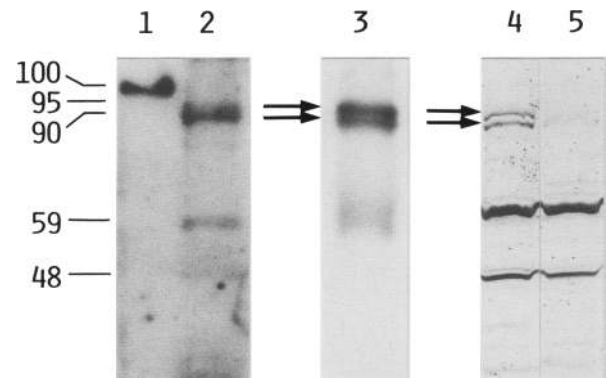
shown to be present in the prenucleolar bodies that participate in nucleolar reformation (Gas et al. 1985; Ochs et al. 1985) and is thus involved in early events of nucleolar function, we wanted to follow the fate of the maternal nucleolin in *Xenopus* oocytes and the expression of nucleolin during embryogenesis. In addition, elucidation of the structure of nucleolin from *Xenopus* would allow us to compare the sequence of this protein in an amphibian with that in rodents.

We report here the isolation and sequence of a cDNA clone encoding *X. laevis* nucleolin. Despite the similarity of these homologous proteins, our data indicate that certain domains of the protein have undergone a different evolution in *Xenopus* and in mammals. We have made use of the cDNA clone to study the expression nucleolin throughout *Xenopus* oogenesis and embryogenesis.

## Results

### Detection and characterization of *X. laevis* nucleolin and its mRNA

IgG purified from a rabbit polyclonal antiserum raised against hamster nucleolin was used to identify nucleolin by immunofluorescence in *Xenopus* hepatocytes and oocytes (Fig. 1). Fluorescent staining was seen in hepatocyte nucleoli and in all amplified oocyte nucleoli. The same antiserum was used to detect the reactive protein by Western blot analysis (Fig. 2). On purified hepatocyte nuclear proteins, the antiserum recognized two polypeptides of  $M_r$  95,000 and 90,000 and gave a weak reaction with two others of  $M_r$  59,000 and 48,000 (Fig. 2, lane 2). In *Xenopus* oocytes from stages I to III (according to Dumont 1972), the antiserum reacted with two polypeptides of  $M_r$  95,000 and  $M_r$  90,000, as in somatic cells, but



**Figure 2.** Detection of nucleolin in *X. laevis*. Protein analysis. (Lanes 1, 2, 4, and 5) Protein extracts separated on a 10% SDS-PAGE and electroblotted were probed using an antiserum raised against CHO nucleolin. Detection of the complexes was achieved either by  $^{125}$ I-labeled protein A (lanes 1 and 2), or by a second antiserum coupled to alkaline phosphatase (lanes 4 and 5). (Lane 3) Protein phosphorylation was obtained with an endogenous kinase contained in a low-ionic-strength extract of *Xenopus* hepatocyte nuclei and addition of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . Proteins were separated as before, then the gel was dried and autoradiographed for 4 hr at  $-70^\circ\text{C}$  with an intensifying screen. (Lane 1) As a control, 0.2  $\mu$ g of nucleolin purified from CHO cells was run in parallel. (Lane 2) Low-ionic-strength extract from nuclei of *Xenopus* hepatocytes. (Lane 4) Protein extract corresponding to 10 oocytes from stage I to III, roughly separated from the yolk (see Materials and methods). (Lane 5) Same as in lane 4, but with oocytes from stage IV to VI. Numbers on the left refer to the molecular weight of the peptides, in kD.

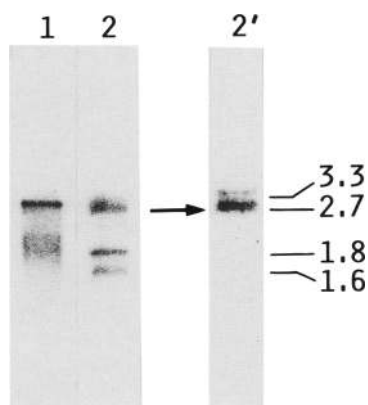
gave only a slight reaction whereas a strong signal was obtained for the two smaller polypeptides (Fig. 2, lane 4). In oocytes from stages IV to VI only these last two polypeptides were observed (Fig. 2, lane 5).

In earlier studies we have shown that a low ionic strength extract of nuclei isolated from Chinese Hamster Ovary (CHO) cells contains a type NII protein kinase able to phosphorylate nucleolin specifically (Caizergues-Ferrer et al. 1987). To further characterize the *Xenopus* protein, we carried out a similar procedure on *Xenopus* hepatocytes. The two polypeptides of  $M_r$  95,000 and  $M_r$  90,000 exhibited the same feature and were labeled with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . A weak labeling of the  $M_r$  59,000 polypeptide was also observed (Fig. 2, lane 3).

To identify the nucleolin mRNA in *Xenopus*, Northern blotting was performed using the heterologous cDNA from hamster CHO cells as a probe (Lapeyre et al. 1985) (Fig. 3). In RNA from CHO cells, the probe reveals an mRNA of 2.7 kb (Fig. 3, lane 1). When *Xenopus* oocyte RNA was probed, three major bands were detected (2.7 kb, 1.8 kb, 1.6 kb) (Fig. 3, lane 2). Further use of a *Xenopus* nucleolin cDNA clone, as a probe on the same blot, showed that the major band of 2.7 kb represents nucleolin mRNA (Fig. 3, lane 2'). One of the related mRNAs revealed by the CHO probe encodes another glycine-dimethylarginine-rich protein (to be published elsewhere). An additional minor band of 3.3 kb also reacted with the *Xenopus* cDNA probe. Further studies to identify the 3.3-kb mRNA are underway.

#### Isolation and characterization of a cDNA specific for *Xenopus* nucleolin

The hamster nucleolin cDNA was used as a probe to



**Figure 3.** Northern blot analysis. RNA was isolated, separated under denaturing conditions in a formaldehyde–1% agarose gel, blotted onto nitrocellulose filter, and then hybridized with different probes. CHO nucleolin cDNA was used to probe total RNA purified either from CHO cells as a control (lane 1) or from *Xenopus* oocytes (lane 2). *Xenopus* nucleolin cDNA was used to probe *Xenopus* oocyte RNA (lane 2').

screen a *Xenopus* oocyte cDNA library constructed in  $\lambda$  gt10 and kindly provided by D. Melton. Fifteen clones were isolated and characterization of five of them showed that they overlapped. The longest cDNA, pXomN1, was 2.1 kb long and was incomplete, lacking the 5' terminus. From its sequence, the sequence of a peptide with 526 residues was deduced (Fig. 4), and a comparison with the 713 residues of the hamster nucleolin sequence (Lapeyre et al. 1987) showed unequivocally that pXomN1 encodes *Xenopus* nucleolin. However, we found that the different domains of the two proteins have undergone very different evolutionary constraints. For instance, two out of the three acidic domains located in the amino half of the protein are much shorter than in rodents, reduced from 25 and 33 contiguous acidic residues to 11 and 20, respectively. Even between different rodents, we have already found that this region is able to evolve very rapidly by replacement (Asp-Glu), deletion, or addition of residues (Lapeyre et al. 1985).

The carboxyl half of the protein contains four RNA-binding domains homologous to those of rodent nucleolin that have been shown to interact with ribosomal RNA (Bugler et al. 1987). The overall organization of the domains is conserved mainly, but two major observations can be made. First, the evolution of each domain is strikingly different between amphibian and rodent with a conservation of 42, 52, 65, and 87% respectively from domain 1 to domain 4, taking only the amino acids that are conserved strictly into account, and allowing some gaps to align the sequence (Fig. 5). The second remark concerns the comparison of the four domains with each other. For instance, in *Xenopus* domains 1 and 4 exhibit only 34% homology, which is less than the homology between the first two domains of *Xenopus* and rodent (42%).

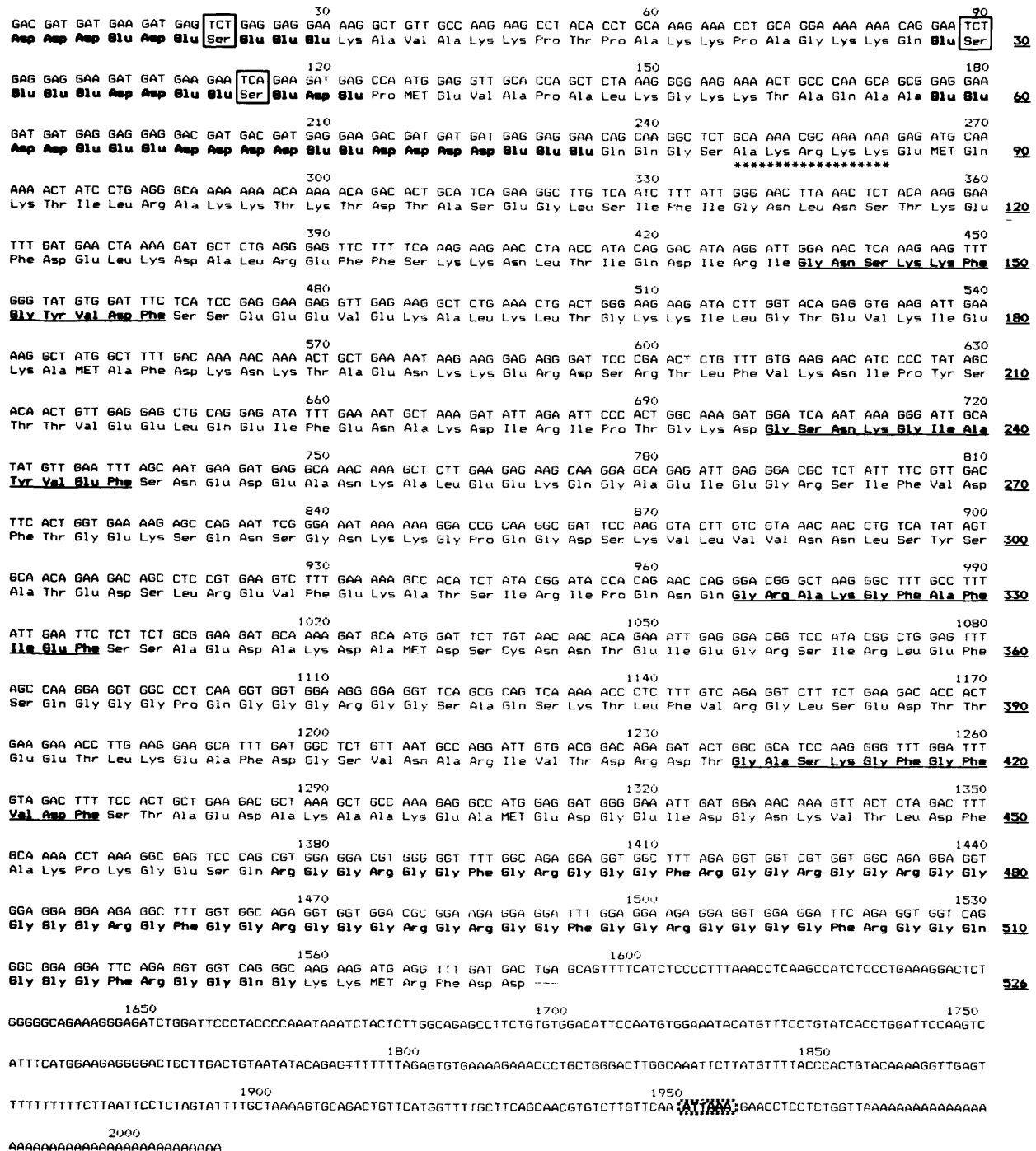
As opposed to the acidic domains, the carboxy-terminal glycine-rich domain is longer in *Xenopus* (61 amino acids) than in CHO (53 amino acids) or in mouse (49 amino acids). The overall composition is roughly conserved, except for the presence in *Xenopus* of two glutamine residues.

#### Nucleolin mRNA accumulation during oogenesis and embryo development

We have followed the accumulation of nucleolin mRNA and the fate of nucleolin during the course of oogenesis and embryogenesis to compare it with the known pattern of accumulation of two classes of ribosomal components, rRNA and r-proteins. The accumulation of nucleolin mRNA during oogenesis and embryogenesis has been analyzed by Northern blot or by RNA dot-blot hybridization. For quantitative data, we have used total RNA instead of poly(A)<sup>+</sup> RNA to avoid any loss of material during oligo(dT)-cellulose column purification. Figure 6 shows the accumulation of RNA during oogenesis and during embryo development. Nucleolin mRNA is accumulated very rapidly at the beginning of oogenesis, reaching the highest value at stage II, after

which it decreases rapidly. This is quite similar to the expression of r-protein mRNAs except these were shown to decrease more slowly after stage II (Pierandrei-Amaldi

et al. 1982). The 2.7- and 3.3-kb mRNAs behave similarly. After fertilization, all transcriptional activity is switched off. From the mid-blastula stage (stage 8)



**Figure 4.** *Xenopus* nucleolin: pXomN1 DNA sequence and deduced amino acid sequence. Numbers above the DNA sequence refer to the nucleotides while numbers underlined on the right refer to the amino acid residues. Acidic tracts are in boldface type. The three serine residues that are candidates for the phosphorylation sites are boxed. The potential nuclear translocation signal, similar to the prototype signal of SV40 large T antigen [Kalderon et al. 1984] is underlined by stars. This signal is located just downstream from the last acidic stretch as in the *Xenopus* protein N038 [Schmidt-Zachmann et al. 1987]. The four RNA-binding consensus sequences, centered on the residues at positions 149, 238, 327, and 417, are in boldface type and underlined. The glycine-rich domain at the carboxyl terminus is in boldface type. The polyadenylation signal is boxed.

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R 1 { X.1. 108  LSIFIGNLNSTKEFDELKDALREFFSKNLTIQDIRIGNSKKGFBYVDFSSEEEVEKALKLTGKKILGTEVKEIKAMAFDKNKTAENKK
      CHO  307  FNLFIGNLNPNKVAELKVAISEPFAKNDLAVVDVRTGTNRKFBYVDFESAEDLEKALELTGLKVFNGNEIKLEK...KGR...DSKK

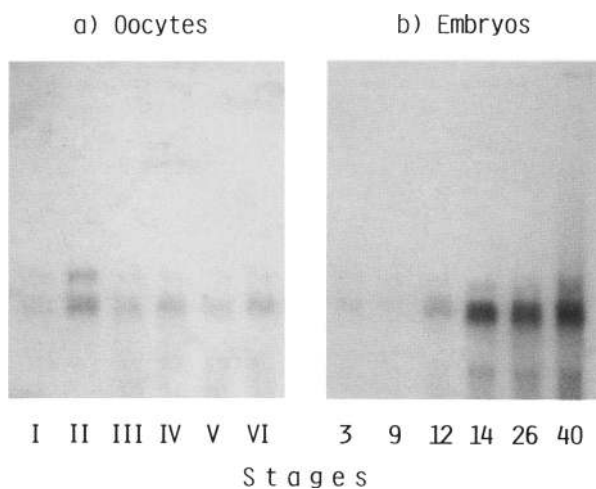
R 2 { X.1. 197  RDSRTLQVKNIPYSTTVEELQEIFENAKDIRIPTGKDSSKKGIAVVEFSNEDEANKALEEKGGAEIEGRS...FVDFTEKESQN...SGNKKGP.QG
      CHO  390  RAARTLLAKNLSFNITEDELKEVFEDALEIRL.VSDDGSKKGIAVIEFKSEADAEKNLEEKGGAEIDGRSVSLY...YTGEKGRQERTG.KNSTWSSG

R 3 { X.1. 288  DSKVLVNNLSYSATEDSLREVFEKATSIRIPQNG.BRKKGFAFIEFSSAEDAKDAMDSCNNTIEGRSIRLEFSQG...GGFQGGGRGSSA
      CHO  483  ESKTLVLSNLSYSATEETLQEVFEKATFIKVPQNGQSKKGBYAFIEFASFEDAKEALNSCNKMEIEGRTIRLEL.QGPRGSPNA...R...SQ

R 4 { X.1. 376  QSKTLFVRGLSEDTTEETLKEAFDGSVNARIVTDRDTSSKKGFBYVDFSTAEDAKAAKEAMEDGEIDGNKVTLDFAKPKGES
      CHO  569  PSKTLFVKGLSEDTTEETLKEAFDGSVNARIVTDRDTSSKKGFBYVDFNSEEDAKAAKEAMEDGEIDGNKVTLDFAKPKGES

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**Figure 5.** Alignment of the four RNA-binding domains of *Xenopus* nucleolin with those of CHO nucleolin (R I to R IV). Numbers on the left refer to the position of the first residue of a line. The consensus sequence indentified previously is in boldface type. Sequences were aligned allowing some gaps (.) to get the best matches (\*).



**Figure 6.** Northern blot showing nucleolin mRNA accumulation during oogenesis and embryogenesis. Total RNA corresponding to 0.5 oocyte or embryo was separated by electrophoresis in a denaturing formaldehyde-1% agarose gel, blotted onto nitrocellulose filter, and hybridized under stringent conditions with nick-translated *Xenopus* nucleolin cDNA as a probe. (a) Stages I to VI of oogenesis according to Dumont 1972. Autoradiography was carried out for 20 hr at  $-70^{\circ}\text{C}$ , with an intensifying screen (XM film from 3M). (b) Stages 3 to 40 of embryogenesis according to Nieuwkoop and Faber (1956). Autoradiography was carried out for 10 hr.

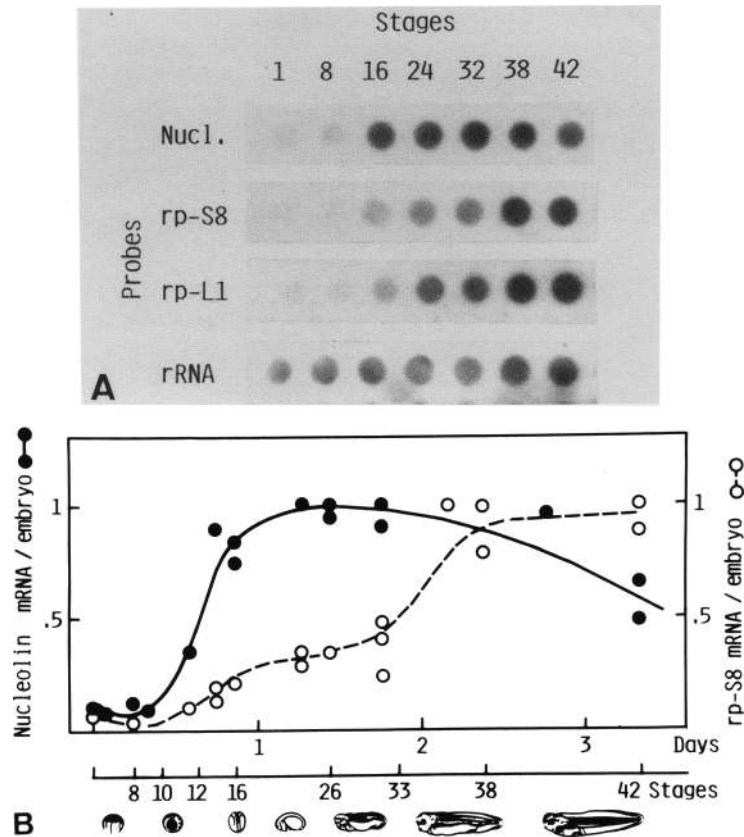
(Nieuwkoop and Faber 1956) there is a revival of transcription for a number of genes, including the nucleolin gene. In embryogenesis a strong increase in the 2.7-kb nucleolin mRNA is observed at stage 14 while 3.3-kb mRNA increases only slightly. We have studied the expression of nucleolin mRNA in comparison with the mRNAs of r-proteins S8 and L1, and with rRNA (Fig. 7A). Quantification of the results of several dot-blots and Northern hybridizations for nucleolin and r-protein is plotted in Figure 7B. Both nucleolin and r-protein mRNAs begin to be transcribed at roughly the same time, but the accumulation patterns are significantly

different. As described already, r-protein mRNA synthesis appears after the mid-blastula transition, increases slowly up to around stage 40, and decreases slightly at the end of embryogenesis (Pierandrei-Amaldi et al. 1982). Nucleolin mRNA increases very rapidly between stages 8 and 14, after which it remains at a constant level up to stage 37 and decreases slowly afterward. These results demonstrate that the accumulation of nucleolin mRNA anticipates both the accumulation of r-protein mRNA and the production of new ribosomes. The highest amount of nucleolin mRNA coincides with the reappearance of nucleoli after gastrula (Hay and Gurdon 1967).

#### *Polysome/mRNP distribution and translation of nucleolin mRNA in Xenopus embryos*

Having established that nucleolin mRNA is accumulated in embryos much more rapidly than r-protein mRNAs or rRNA, we have studied the fate of this mRNA by analyzing its distribution in polysomes and free mRNPs. It appears that, as early as stage 18, about 60% of nucleolin mRNA is loaded onto polysomes, although only 25% of r-protein mRNAs are engaged. At stage 36, 80% of nucleolin and 60% of r-protein S8 messages are translated. Therefore, nucleolin mRNA not only is accumulated before that of the r-proteins but also is used for translation earlier (Fig. 8).

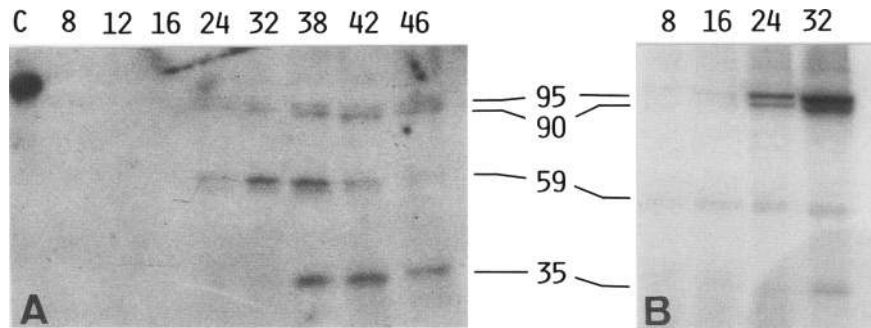
Finally, we looked for the appearance of the protein during embryogenesis. At the end of oogenesis, we have shown that using the nucleolin antiserum on Western blots only the  $M_r$  59,000 and  $M_r$  48,000 polypeptides were detected (Fig. 2, lane 5). During embryogenesis (Fig. 9A), the two polypeptides of  $M_r$  95,000 and  $M_r$  90,000 were detected from stage 24 and present until stage 46. It is even possible to detect nucleolin synthesis earlier, at stage 16, by using its capacity for endogenous phosphorylation (Fig 9B). The  $M_r$  59,000 peptide appears from stage 16 when a weak revival of rDNA gene transcription occurs, but it is particularly prominent at stage 32 and 38 when ribosome accumulation is very high (Pierandrei-Amaldi et al. 1982) and then decreases until stage



**Figure 7.** Relative expression of rRNA, nucleolin, and r-protein mRNAs during embryogenesis. (A) Total RNA corresponding to two embryos of each stage was analyzed by dot-blot and hybridization with different DNA probes: nucleolin (*Nucl.*), two r-proteins (*rp-S8* and *rp-L1*), and rDNA (*rRNA*). (B) Quantitative data were obtained for nucleolin and r-protein S8 by densitometric scanning of the autoradiograms, the values from four independent experiments have been plotted. Average values for each stage are expressed in percent of the value of the stage with the highest score. rpS8 mRNA (O-O); nucleolin mRNA (●-●).

Probes	Stages	gradient regions		% mRNA on polysomes
		polysomes	80S   mRNP	
Nucl.	18			62
	36			82
rp-S8	18			25
	36			60

**Figure 8.** Polysome/mRNP distribution of nucleolin and r-protein mRNA. Cytoplasmic fractions of 15 embryos of stage 18 and 36 were run through sucrose gradients. RNA was extracted from gradient fractions and analyzed by dot-blot hybridization with probes for nucleolin, or for r-protein (S8) mRNA. The percent of mRNA in polysomal or mRNP fraction was determined by densitometric scanning of the autoradiogram.



**Figure 9.** Fate of nucleolin during embryogenesis. Total protein extracts of 10 embryos were prepared in the presence of protease inhibitors as described in Materials and methods. (A) Proteins corresponding to one embryo per lane were electrophoresed on a 10% SDS-PAGE. The amount of protein per lane increases from 10  $\mu$ g for stage 8 to 100  $\mu$ g at stage 46. The gel was blotted onto nitrocellulose, probed with purified IgG directed against nucleolin of CHO, then stained with  $^{125}$ I-labeled protein A. The filter was exposed for 20 hr at  $-70^{\circ}\text{C}$  with an intensifying screen. Lanes: (C) Control lane, 0.2  $\mu$ g of purified CHO nucleolin; (8–46) embryo stage according to Nieuwkoop and Faber (1956). (B) Endogenous phosphorylation of nucleolin in embryonic extracts. Same protein extracts as in A were incubated in the presence of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . Proteins were separated on a 10% SDS-PAGE. The gel was dried and autoradiographed for 4 hr at room temperature. (Lanes 8–32) Embryo stages. Numbers in the middle refer to the peptides in kD.

46. Another peptide of  $M_r$  35,000 that was not detected in oocytes is accumulated from stage 38 and remains constant up to stage 46.

## Discussion

### Structure of nucleolin in *X. laevis*

Our results obtained with an antiserum against hamster nucleolin and with cDNA cloning show that a protein highly homologous to rodent nucleolin is present in *X. laevis*. The overall organization of the protein is essentially the same in *Xenopus* and in rodent, with three distinct domains: an acidic amino-terminal region, four RNA-binding domains, and a glycine-rich carboxyl terminus. Extended clusters of negatively charged amino acids are found to be responsible for the abnormal mobility of protein in SDS-PAGE (Kaufmann et al. 1984; Kleinschmidt et al. 1986). Since rodent nucleolin,  $M_r$  77,000, migrates with an apparent molecular weight of 100,000, we can deduce that the fewer acidic residues in *Xenopus* can reflect the difference in apparent molecular weight between rodent and amphibian. The presence of two molecular forms of  $M_r$  100,000 and 95,000 was reported in rodent (Bourbon et al. 1983), and the  $M_r$  95,000 and 90,000 polypeptides found in *Xenopus* could correspond to these. Similar acidic domains have been described already in other nuclear proteins: *Xenopus* nuclear protein NO38 (Schmidt-Zachmann et al. 1987), the karyophilic protein N1/N2 (Kleinschmidt et al. 1986), nucleoplasmin (Bürglin et al. 1987; Dingwall et al. 1987), the HMG 1 and 2 proteins (Walker 1982), and several other proteins (for review, see Earnshaw 1987). Nucleoplasmin and protein N1/N2 are involved in nucleosome assembly (Dilworth et al. 1987), although, on the contrary, we have shown that nucleolin can induce chromatin decondensation by displacement of histone H1 (Erard et al. 1988), as can also be achieved by HMG 1 or 2 proteins. In both cases, polyacidic stretches are the

obvious candidates for histone binding. These stretches could also be involved in preribosome formation by interacting with positively charged ribosomal proteins. Indeed, nucleolin that is already bound to the pre-rRNA (see below) could neutralize the charge of ribosomal proteins, allowing them to associate in nascent preribosomes.

The mechanism of specific RNA sequence recognition by nucleolin is still an open question. We have found, both in vivo and in vitro (Bourbon et al. 1988b), that nucleolin interacts with three different classes of sites along the pre-rRNA molecule, according to their binding affinities. Some of these sites, located in 18S and in 28S RNAs, correspond to very well conserved sequences, while others, located for example in the external spacer, exhibit very poor conservation between different species. Thus, a major point to address is to know how the four RNA-binding blocks work. A first hypothesis is that they act in an independent manner with, for instance, the strongly conserved fourth block interacting with a well-conserved sequence in 18S, although the less-conserved first block that has evolved very rapidly could recognize variable sequences in the external spacer and reflect the coordinated evolution of both the protein and of the target RNA. A second hypothesis could be that the four blocks work in a coordinate manner with all of them involved in each interaction, the most conserved responsible for the specificity of recognition while the others would only cooperate, with a decreasing specificity from the third to the first domain. In vitro studies using the different RNA-binding domains produced by genetic engineering and synthetic RNAs are in progress to test these hypotheses.

The long, flexible, glycine-rich domain is conserved roughly between rodents and *Xenopus*. This sequence is also found in fibrillarin, a nucleolar protein which is associated with U3 RNA (Lischwe et al. 1985), and also in different hnRNPs (Chung and Wooley 1986; Cobianchi

et al. 1986). This domain could be involved in RNA binding through dimethylarginine residues or, by analogy with keratin and intermediate filaments, be involved in protein-protein interactions (Fisher et al. 1986). Nucleolin and fibrillarin that are both present in the prenucleolar bodies (Gas et al. 1985; Benavente et al. 1987) could interact either together or with other proteins and play a role in nucleolar reformation.

#### *Nucleolin is involved in the first steps of ribosome biogenesis*

In *Xenopus* oocytes, after rDNA amplification, a large number of nucleoli is formed (400 to 2500 per oocyte), followed by a huge accumulation of ribosomes from stage III. Nucleolin mRNA is accumulated in stage II, but the nucleolin detectable in earlier oocytes appears to be matured rapidly to smaller polypeptides of  $M_r$  59,000 and 48,000. We interpret these results as indicating a concomitant and related maturation of nucleolin with the transcription of rDNA. This is in agreement with our previous results obtained in vitro when studying transcription in isolated nucleoli. Nucleolin acts as an inhibitor of pre-rRNA synthesis when its proteolysis is inhibited. In the absence of a protease inhibitor, maturation of phosphorylated nucleolin occurs and transcription of rDNA is allowed (Bouche et al. 1984). Maturation of nucleolin also has been observed under other physiological conditions: (1) phosphorylation of nucleolar protein in rat ventral prostate is stimulated after testosterone or polyamine treatment and is followed by an increased degradation of nucleolin to a  $M_r$  59,000 peptide (Suzuki et al. 1985); (2) hyperphosphorylation of a  $M_r$  60,000 peptide structurally and immunologically related to nucleolin appears after tumor promoter treatment of human fibroblast (Issenger et al. 1988). In vitro maturation of nucleolin has been described that results in peptides of  $M_r$  95,000, 70,000 and 60,000 in CHO cells (Bourbon et al. 1983) and in a  $M_r$  48,000 peptide in calf thymus (Sapp et al. 1986).

After oocyte maturation the germinal vesicle breaks down and the extra nucleoli vanish. Then, from mid-blastula transition, nucleolin mRNA is accumulated and recruited onto polysomes more efficiently than r-protein mRNAs. Nevertheless, an increased recruitment of both mRNAs onto polysomes between the neurula and tailbud stages suggests the existence of a translational control, similar to the one described for r-proteins (Pierandrei-Amaldi et al. 1985). Nucleolin protein is detected as early as stage 16 when the nucleoli reform; its accumulation precedes r-protein synthesis and ribosome accumulation, known to become very active after the tailbud stage (stage 26). From this stage, nucleolin begins to mature and smaller peptides that react with the anti-serum are detected in later embryonic stages. These results, along with our in vitro experiments, suggest that in vivo nucleolin is somehow involved in the preparation and/or preliminary steps of ribosome synthesis.

The results reported here support the notion that nu-

cleolin performs vital functions in ribosome biogenesis, via different domains within the protein molecule. Furthermore, we present evidence that the maturation of nucleolin is linked closely to these functions.

## Materials and methods

### *Biological materials*

*X. laevis* were purchased from Fisch Hoeck, South Africa, or from SEREA, CNRS, France. Oocytes were separated manually into six stages according to Dumont (1972) and staging of embryos was according to Nieuwkoop and Faber (1956).

### *Cell fractionation*

*X. laevis* hepatocyte nuclei were purified as described previously (Mathieu et al. 1975). For polysome purification, 15 embryos from each stage were homogenized in 0.5 ml of 10 mM Tris-Cl (pH 7.4), 150 mM KCl, 4 mM MgCl<sub>2</sub>, 0.05% Triton X-100. The cytoplasmic extracts were loaded onto 15–50% sucrose gradients. Centrifugation was performed on a Spinco centrifuge with a SW41 rotor for 2 hr at 37,000 rpm. Gradient fractions were ethanol precipitated overnight at  $-20^{\circ}\text{C}$ , and the RNA was extracted from the precipitates (Pierandrei-Amaldi et al. 1982).

### *Immunofluorescence microscopy*

Isolated liver nuclei were cytocentrifuged onto polylysine-coated microscope slides. They were fixed with 4% paraformaldehyde in phosphate buffer (pH 7.2) for 15 min. After washing, nuclei were processed for immunofluorescence microscopy as described previously (Gas et al. 1985). Small pieces of ovaries were removed and fixed as above. After washing, samples were quick-frozen in isopentane cooled with liquid nitrogen. Cryostat sections of 12- $\mu\text{m}$  thickness were air-dried and processed for immunofluorescence as above.

### *Protein analysis*

Low ionic strength extracts of *Xenopus* hepatocyte nuclei were purified as described previously (Bourbon et al. 1983). Protein extracts from one to ten oocytes or embryos were homogenized in 50 to 100  $\mu\text{l}$  of 10 mM Tris-Cl (pH 7.6), 50 mM NaCl, 2 mM EDTA and 5  $\mu\text{g/ml}$  leupeptin, 5  $\mu\text{g/ml}$  aprotinin, 2  $\mu\text{g/ml}$  pepstatin, 10 mM benzamide-Cl, 1 mM phenylmethylsulfonyl-fluoride (PMSF) as protease inhibitors. After a 30-min RNase B treatment (100  $\mu\text{g/ml}$ ), yolk and lipids were removed by two cycles of centrifugation at 10,000g for 10 min at  $4^{\circ}\text{C}$ . Proteins were precipitated by ammonium sulfate: nucleolin and related peptides precipitate between 40 and 80% of saturation. Protein molecular weight markers were obtained from BioRad. Proteins were separated on 10% SDS-PAGE according to Laemmli (1970). Proteins were transferred to nitrocellulose by electroblotting using a BioRad apparatus in 25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol (pH 8) for 2 hr at 60 V (0.3 A) at  $4^{\circ}\text{C}$ . For immunostaining of the Western blot, we used a protocol described previously (Lapeyre et al. 1985).

### *RNA analysis*

Total RNA from oocytes or embryos, or RNA from the polysomal fraction was extracted according to Probst et al. (1979) and analyzed either by dot-blot or Northern hybridization as



described previously (Pierandrei-Amaldi et al. 1982). The  $^{32}\text{P}$ -labeled DNA probes were obtained by nick-translation. Prehybridization and hybridization were performed in 50% formamide at 42°C as described by Thomas (1980). Filters were then washed with two changes of  $2 \times \text{SSC}$ , 0.1% SDS at room temperature and two changes of  $0.1 \times \text{SSC}$ , 0.1% SDS at 48°C and exposed to X-ray film.

#### Quantification of autoradiograms

LKB Ultrosan XL laser was used for densitometric analysis of X-ray film of RNA dot blot and Northern blots.

#### Screening of the cDNA bank, subcloning, and sequencing

A cDNA bank constructed by D. Melton in  $\lambda$  gt10 with poly(a)<sup>+</sup> RNA from *Xenopus* oocytes was screened using the insert of plasmid BL16.2 (Lapeyre et al. 1985) as a specific probe for nucleolin. The isolated recombinant phages were characterized and inserts were subcloned in pTZ 19R. Nucleotide sequencing was performed according to Maxam and Gilbert (1980) with the addition of a T-specific reaction (Rubin and Schmid 1980) or using the dideoxy chain termination method of Sanger et al. (1977). DNA sequence was determined on both strands and across most restriction sites used as initiation points.

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#### Note

Sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries.

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