The Ribosomal RNA Processing Machinery Is Recruited to the Nucleolar Domain before RNA Polymerase I during *Xenopus laevis* Development

Céline Verheggen,* ‡ Geneviève Almouzni,‡ and Danièle Hernandez-Verdun*  
*Institut Jacques Monod, UMR 7592, 75251 Paris, France; and ‡Institut Curie, Section de Recherche, UMR 144, 75248 Paris, France

**Abstract.** Transcription and splicing of messenger RNA s are temporally and spatially coordinated through the recruitment by RNA polymerase II of processing factors. We questioned whether RNA polymerase I plays a role in the recruitment of the ribosomal RNA (rRNA) processing machinery. During *Xenopus laevis* embryogenesis, recruitment of the rRNA processing machinery to the nucleolar domain occurs in two steps: two types of precursor structures called prenucleolar bodies (PNBs) form independently throughout the nucleoplasm; and components of PNBs I (fibrillarin, nucleolin, and the U3 and U8 small nucleolar RNAs) fuse to the nucleolar domain before components of PNBs II (B23/NO38). This fusion process is independent of RNA polymerase I activity, as shown by actinomycin D treatment of embryos and by the lack of detectable RNA polymerase I at ribosomal gene loci during fusion. Instead, this process is concomitant with the targeting of maternally derived pre-rRNAs to the nucleolar domain. A bsence of fusion was correlated with absence of these pre-rRNAs in nuclei where RNA polymerase II and III are inhibited. Therefore, during *X. laevis* embryogenesis, the recruitment of the rRNA processing machinery to the nucleolar domain could be dependent on the presence of pre-rRNA s, but is independent of either zygotic RNA polymerase I transcription or the presence of RNA polymerase I itself.

**Key words:** prenucleolar body • nucleologenesis • pre-rRNA • RNA polymerase I transcription • *Xenopus laevis* development

**Introduction**

Defining the coordinating events during RNA transcription and processing is becoming increasingly important in the context of their regulatory role in gene expression and nuclear organization (for reviews see Lamond and Earnshaw, 1998; Misteli and Spector, 1998). It has been reported that messenger RNA (mRNA) transcription and processing is coordinated by the recruitment of processing factors to transcription sites by RNA polymerase II (RNA pol II) (Jiménez-Garcia and Spector, 1993; Misteli et al., 1997; Bentley, 1999; Misteli and Spector, 1999). Remarkably, the activation of ribosomal gene (rDNA) transcription at the end of mitosis is also accompanied by the recruitment of processing complexes (Scheer and Benavente, 1990; Thiry and Gossens, 1996). This therefore raises the issue of whether there is a link between active transcription and processing for ribosomal RNA (rRNA).

Processing of rRNA s involves cleavage, methylation, and pseudouridylation of the primary rRNA s (Hadjiolov, 1985; Smith and Steitz, 1997). Cleavage is controlled by several ribonucleoprotein (RNP) complexes that act in an ordered manner to remove the external transcribed spacers (5′ETS and 3′ETS) and the internal transcribed spacers (ITS1 and ITS2). Fibrillarin (Ochs et al., 1985b) and nucleolin (Ginisty et al., 1998) associated with several small nucleolar RNAs (snoRNAs), including U3, could play a role during the first steps of rRNA processing (for a review see Tollervey, 1996). Subsequent cleavages involve endoribonuclease activities such as the MRP RNase complex (Lygerou et al., 1996a,b; Dichtl and Tollervey, 1997; Pluk et al., 1999; van Enenlema et al., 1999) for the ITS1, and protein B23 (Savkur and Olson, 1998) and U8 (Michot et al., 1999) for the ITS2. In *Xenopus laevis*, U8 was shown to be involved both in early 3′ETS and late ITS2 cleavages.

**Address** correspondence to D. Hernandez-Verdun, Institut Jacques Monod, 2 place Jussieu, 75251 Paris Cedex 05, France. Tel.: 33 1 44 27 40 38. Fax: 33 1 44 27 59 94. E-mail: dhernand@ccr.jussieu.fr

**Abbreviations used in this paper:** Br-UTP, 5-bromouridine-5′-triphosphate; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; ETS, external transcribed spacers; FISH, fluorescent in situ hybridization; ITS, internal transcribed spacers; MBT, midblastula transition; mRNA, messenger RNA; PNB, prenucleolar bodies; rDNA, ribosomal gene; rRNA, ribosomal RNA; RNA pol I; RNA polymerase I; snoRNA, small nucleolar RNA.
Several proteins of the rRNA processing machinery are detected in prenucleolar bodies (PNBs; Ochs et al., 1985a), which are scattered throughout the nucleus in early G1 (Benavente et al., 1987; Jiménez-Garcia et al., 1989; Ochs and Smetana, 1991; A zum-Gélade et al., 1994; Scheer and Weißenberger, 1994; B even et al., 1996; D undr et al., 1997; Zatspena et al., 1997). Fibrillarin, nucleolin, Nop52, PM-Scl 100/exosome, and protein B23 are found within these PNBs, as are the U3 and U14 snoRNA s (A zum-Gélade et al., 1994; Gautier et al., 1994; Jiménez-Garcia et al., 1994; B even et al., 1996; Mitchell et al., 1997; Fomproix and Hernández-V erdún, 1999; Savino et al., 1999). Therefore, the PNBs appear to contain preassembled nucleolar complexes mainly involved in processing steps of the pre-rRNA s (Scheer and Weißenberger, 1994). Distinct PNBs are involved in the delivery of specific processing complexes to the nucleolar domain (Fomproix and Hernández-V erdún, 1999; Savino et al., 1999). Since the delivery of the different PNBs follows a temporal order, it has been proposed that reassembly into nucleoli could proceed by a stepwise mechanism that reflects the role of these complexes (Savino et al., 1999). The temporally regulated targeting of PNBs during the cell cycle thus appears dependent on the constituents of these PNBs. However, the involvement of the transcription and/or the transcripts in this process is unknown.

Remarkably, during early X. laevis embryogenesis, a unique situation was revealed in which regroupment of fibrillarin and nucleolin around the rDNA occurred before the apparent activation of rRNA pol I-dependent transcription (V erheggen et al., 1998). The first cell cycles of X. laevis embryogenesis provide an interesting biological situation since transcription is established de novo after 12 synchronized cell cycles devoid of transcription (Brown and Litton, 1964; Newport and Kirschner, 1982). At the midblastula transition (MBT), RNA pol II- and III-dependent transcription is activated, whereas RNA pol I transcription is initiated later (Shikokawa et al., 1981a; Newport and Kirschner, 1982). This biological situation makes it possible to study PNB assembly and delivery in the context of active or inactive RNA pol I transcription. Before MBT, scattered PNBs containing fibrillarin exhibit similar ultrastructural features to postmitotic PNBs and at MBT fibrillarin regroups around the rDNA with maternal pre-rRNA s (V erheggen et al., 1998). At MBT, the association of RDNAs with UB F was demonstrated (Be ll et al., 1997; V erheggen et al., 1998), but the presence of other partners of the transcription machinery and, in particular, the RNA pol I complex is not yet established. Indeed, at MBT it was reported that RNA pol I accumulated in nucleoplasmic structures different from PNBs (Bell and Scheer, 1999), without information on its association with rDNA.

Nuclei assembled in X. laevis egg extracts contain PNB-like structures with fibrillarin, nucleolin, Nop52, protein B23 (NO 38 in X. laevis), U3, and U8 (Bell et al., 1992; Bauer et al., 1994; Bell and Scheer, 1997). Since these PNBs assembled in vitro were not observed to fuse into a nucleolus, a reasonable hypothesis was proposed that this lack of nucleolar assembly is due to the absence of transcription in this system (Bell et al., 1992).

In the present study, we demonstrate that during both X. laevis embryogenesis and in nuclei assembled in vitro, two types of PNBs containing components of the rRNA processing machinery exist. During X. laevis embryogenesis, the recruitment of both types of preassembled complexes to the nucleolar domain occurs at a time when the RNA pol I complex is not detected in the nucleolar domain. Furthermore, this recruitment is not dependent on RNA pol I activity, but correlates precisely with the presence of pre-rRNA s of maternal origin. Pre-rRNA s are absent from nuclei in which RNA pol II and III transcription was inactive and, in this case, recruitment of the rRNA processing machinery does not occur.

Materials and Methods

Primary Antibodies and Probes

A ntibodies with the following specificities were used: a human auto-immune serum directed against fibrillarin (Gautier et al., 1994); a rabbit polyclonal serum directed against human nucleolin (a kind gift of C. Faucher, LBME, CNRS, Toulouse, France); a monoclonal culture supernatant recognizing the X. laevis nucleolar protein NO 38, a homologue of the mammalian nucleolar protein B23 (No-63; Schmidt-Zachmann et al., 1987); and a mouse monoclonal ascites fluid recognizing the X. laevis RNA pol I complex (a kind gift of M. Schmidt-Zachmann, DKFZ, Heidelberg, Germany).

The rDNA probe for rRNA detection corresponds to the entire X. laevis ribosomal transcription unit inserted into pBluescript II KS+ (a gift from B. St. John, University of Wisconsin, Madison, WI). Probes and oligonucleotides were obtained from B. St. John (University of Wisconsin, Madison, WI). Probes and oligonucleotides were obtained from Biodepti (Biodepti, Inc., Madison, WI).

Assembly of Nuclei in X. laevis Egg Extracts

Eggs were obtained from female X. laevis and interphase low-speed egg extracts were prepared as previously described (A Imouzi, 1998). This crude extract can be maintained on ice for 4 h without appreciable decay of nuclear assembly activity. Demembraned X. laevis sperm nuclei were prepared and permeabilized with lysolysin (A Imouzi, 1998). For nuclear assembly, 103 sperm heads were added to 100 μl of egg extract and maintained at 23°C. A fter 45 min, the amount of sperm heads was determined by light microscopy.
blistomeres was required for drug accessibility, embryos 3 h 30 min after fertilization were transferred to Ca²⁺- and Mg²⁺-free medium containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, and 7.5 mM Tris HCl, pH 7.6, as previously described (Duval et al., 1990). A cytokinase D (Sigma Chemical Co.) was added to the incubation medium at the desired concentration from a stock solution at 5 mg/ml. A s a control, embryos were incubated in Ca²⁺- and Mg²⁺-free medium without actinomycin D. Other drugs, such as α-amanitin, K⁺-dichloro- p-o-ribofuranosylbenzimidazole (DRB; Sigma Chemical Co.) could not be used as inhibitors of transcription in X. laevis embryos. When α-amanitin was added to the incubation medium, it did not penetrate into the dissociated blastomeres and DRB had a deleterious effect on the division of the embryos. Suspensions of embryonic nuclei were prepared from embryos taken at precise times after fertilization as described (Verheggen et al., 1998).

**Immunofluorescence and In Situ Hybridization**

In vitro reconstituted nuclei and suspensions of embryonic nuclei were fixed with 1 vol 4% paraformaldehyde in PBS. The suspensions of fixed nuclei could be stored several weeks at 4°C. For immunofluorescence and in situ hybridization studies, nuclei were centrifuged onto a coverslip. After washing, the coverslips were postfixed in methanol, paraformaldehyde in 0.1% Triton X-100 (iBi) in PBS, and rinsed.

For immunofluorescence labeling, the coverslips were incubated with primary antibodies, followed by FITC- or TRITC-conjugated secondary antibodies (anti-human, anti-mouse, or anti-rabbit IgG; Jackson ImmunoResearch Laboratories) or cy3-conjugated anti-mouse antibodies (Sigma Chemical Co.), rinsed, counterstained with DAPI (4'-6-diamidino-2-phenylindole dihydrochloride; Polysciences, Inc.) and mounted with an anti-fading mounting medium (Citifluor).

**Electron Microscopy**

In vitro reconstituted nuclei were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 4°C. They were washed in cacodylate buffer, postfixed in 1% O₃SO₄ for 1 h at 4°C, dehydrated in alcohol, and embedded in Epon 812. Ultrathin sections were contrasted with uranyl acetate for 1 h and lead citrate for 2 min, and examined in a Philips EM412 electron microscope.

**In Situ Transcription Assay**

To localize transcription sites, 5-bromouridine-5'-triphosphate (Br-UTP) incorporation into embryonic nuclei was performed as previously described (Verheggen et al., 1998). The rRNA hybridization mixture contained 40% formamide (GIBCO BRL), 10% (w/v) ddCTP, dextran sulfate (Sigma Chemical Co.), 50 ng/ml sonicated salmon testes DNA (Sigma Chemical Co.), and the biotinylated rRNA probe diluted to a final concentration of 1 ng/ml in 2× SSC. For the detection of U3 and U8, the hybridization mixture contained 30% formamide, 10% (w/v) dextran sulfate, 50 ng/ml sonicated salmon testes DNA, and the 3' end biotinylated oligonucleotides complementary to U3 and U8 diluted to a final concentration of 2 ng/ml in 2× SSC. As a control for the detection of rRNA hybridization was preceded by RNase digestion as described (Highett et al., 1993).

**Results**

**Components of the rRNA Processing Machinery Are Located in Two Types of PNBs in Early Embryonic and Reconstituted Nuclei**

During the building process of the nucleolus in X. laevis, embryonic nuclei, components of rRNA processing machinery are associated in bodies called PNBs dispersed in the nucleoplasm. A first class of PNBs (PNBs I) containing fibrillarin and nucleolin, is observed 6 h after fertilization. A maternal pool of U3 is maintained during early development of X. laevis (Xia et al., 1995) and we compared its distribution to fibrillarin in embryonic nuclei 6 h after fertilization. Fluorescent in situ hybridization (FISH) of U3 snoRNAs was performed after immunolabeling of fibrillarin. U3 snoRNAs were colocalized with fibrillarin in all PNBs I and were also present on some other dots in the nucleoplasm (Fig. 1, a–d). Moreover, U8 snoRNAs, implicated both in early and late steps of rRNA processing in X. laevis, were also detected in PNBs I by FISH (see Fig. 3).

PNBs I can be the site of pre-rRNA processing, did not colocalize with fibrillarin when both proteins were revealed on embryonic nuclei isolated 6 h after fertilization (Fig. 1, e–h). Instead, B23/NO38 appeared diffuse in the nucleoplasm and some proteins were distributed in numerous small dots throughout the nucleoplasm, and were termed PNBs II. This could be an intermediate step in the recruitment of B23/NO38 to PNBs II since later, B23/NO38 becomes distributed in PNBs II of large size (see Fig. 4). Consequently, the formation of PNBs II appears to be delayed, compared with PNBs I.

The recruitment of nucleolar proteins and snoRNAs of the rRNA processing machinery to fibrillar PNB-like structures has been described using in vitro reconstituted nuclei (Bell et al., 1992; Bauer et al., 1994). We performed double immunolabeling experiments on reconstituted nuclei to see whether the components of the processing machinery were in the same structures or in different classes of PNBs in embryonic nuclei. Permeabilized sperm nuclei incubated in a low-speed X. laevis egg extract formed largely decondensed nuclei. A fter immunolabeling of fibrillarin, U3 and U8 snoRNAs were detected by FISH in these reconstituted nuclei. U3 was colocalized with fibrillarin in large round-shaped structures (Fig. 1, i–l). U8 displayed a similar distribution (not shown). B23/NO38 did not colocalize with fibrillarin (Fig. 1, m–p), but showed a distribution similar to that of the embryonic nuclei 6 h after fertilization. In particular, B23/NO38 accumulated in small and numerous dots. In EM, two types of nucleolar bodies were observed (Fig. 2). In reconstituted nuclei, the general view of sections makes it possible to discriminate few dense structures and numerous small nuclear bodies (Fig. 2 a). The dense bodies (mean diameter, 0.5 μm) exhibited a fibrillar matrix containing densely packed granules of 10–15 nm (Fig. 2 b), structures also observed at premBT in embryonic PNBs (data not shown). Their size, distribution, and morphological features are compatible with the identification of these bodies as PNBs I. On the contrary, the small nuclear bodies (mean diameter, 0.1 μm) were gray without visible dense granules (Fig. 2 c). Based on their size, distribution
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(Fig. 2 a), and number, we postulated that these nuclear bodies might correspond to PNBs I. Small nuclear bodies with low contrast were also observed in embryonic nuclei (data not shown). Immunolabeling using B23/NO38 antibody was attempted, but without success, most probably because for EM, the antibody has too low a titer. Thus, morphologically different nuclear bodies were formed in reconstituted nuclei. We propose that they correspond to PNBs I and PNBs II.

PNBs I and PNBs II Are Targeted to the Nucleolar Domain with Different Kinetics during Development

We next defined the kinetics of recruitment of PNBs I and PNBs II to the nucleolar domain. Embryonic nuclei were isolated at various times during development, between 7 and 9 h after fertilization. Labeling of PNB I components (fibrillarin, nucleolin, U3, and U8) revealed a progression towards regrouping in nucleolar domain. PNBs I were first seen gathered in two restricted areas of the nucleoplasm in some nuclei 7 h after fertilization (Fig. 3, a–c). By 9 h after fertilization, they had fused in two large nucleolar domains in most nuclei (Fig. 3, d–f). Intermediate structures larger than PNBs I and smaller than the nucleolar domain were formed (Fig. 4 b) and were visible by phase-contrast microscopy (Fig. 4 d) in nuclei between 7 and 9 h after fertilization. B23/NO38 colocalized with fibrillarin in these structures (Fig. 4 c). Thus, fusion of the PNB I and PNB II components to the nucleolar domain was initiated nearly...
at the same time. In contrast, large amounts of B23/NO38 remained in PNBs II in the nucleoplasm, even when all PNBs I had completely fused (Fig. 4 g). PNBs II in nuclei 7 (Fig. 4 a) and 9 h (Fig. 4 e) after fertilization were larger than in nuclei 6 h after fertilization (Fig. 1). Complete targeting of B23/NO38 to the nucleolar domain was only observed in nuclei 11 h after fertilization (Fig 4 i). Targeting of B23/NO38 to the nucleolar domain was thus achieved at a later time, compared with the other rRNA processing components found in PNBs I. Similar delay between B23 and fibrillarin targeting was also observed in early G1 X. laevis A6 cells (data not shown).

Fusion of PNB components to the nucleolar domain does not occur in in vitro reconstituted nuclei after several hours of incubation in the egg extract. B23/NO38 distribution remained diffuse and in small PNBs, as in embryonic nuclei 6 h after fertilization. Therefore, we attempted to identify the events that occur in embryonic nuclei and not in reconstituted nuclei that could be responsible for targeting of the rRNA processing machinery to the nucleolar domain.

**RNA Polymerase I Was Excluded from the Nucleolar Domain during the Recruitment of Maternally Derived Pre-rRNAs and their Processing Machinery**

It is generally accepted that the onset of rDNA transcription in cultured cells at the end of mitosis is an event re-
quired for relocalization of the rRNA processing machinery to the nucleolar domain (Scheer et al., 1993). RNA pol I could play a role in this recruitment process. This prompted us to investigate the distribution of RNA pol I at the time of nucleolar assembly in *X. laevis* embryos.

Immunolabeling of nuclei between 7 h and 11 h after fertilization with an mAb that immunoprecipitates the *X. laevis* RNA pol I complex (M. Schmidt-Zachmann, personal communication) revealed a speckled distribution (Fig. 5), as recently described (Bell and Scheer, 1999). rRNAs were previously detected in the nucleolar domain at the time of PNB gathering and were identified as 40S transcripts, probably derived from a maternal pool (Verheggen et al., 1998). We performed in situ hybridization of rRNAs on nuclei previously labeled with the antibody against RNA pol I, to simultaneously visualize the RNA pol I complex and the pre-rRNAs on the nucleolar domain in nuclei isolated between 7 (Fig. 5, a–c) and 9 h after fertilization (Fig. 5, d–f). Speckle-like structures containing RNA pol I were far from nucleolar domains and consequently from rDNAs. This indicated that pre-rRNAs were maternally derived. A fraction of RNA pol I was found associated with the nucleolar domain only in nuclei 11 h after fertilization (Fig. 5, g–i). This corresponded precisely with the time when RNA pol I transcription began to be observed by the in situ transcription assay (see below).

**Zygotic Pre-rRNA Is Not Required for the Recruitment of the Maternal Pre-rRNAs and Processing Machinery to the Nucleolar Domain**

To confirm that zygotic nascent pre-rRNAs, even in undetectable amounts, were not present in the newly formed nucleolar domain between 7 h and 9 h after fertilization, embryos were treated with low doses of actinomycin D to inhibit RNA pol I transcription. To allow the drug to diffuse in all the embryonic cells, actinomycin D was diluted in Ca²⁺- and Mg²⁺-free medium, conditions that dissociate
its sensitivity to and actinomycin D–treated embryos, was characterized by Br-UTP incorporation in isolated nuclei from control 12 h after fertilization and in situ transcription assays in RNA pol I transcription. This prompted us to use embryos D when applied to whole embryos to selectively block gastrulation movements were abolished.

At this time, divisions still occurred, but blastomeres nor the presence of actinomycin D appeared changes in the transcription pattern during development. Transcription observed 9 h after fertilization was inhibited by α-amanitin, indicating that it corresponds to RNA pol II and/or III activities (Fig. 8 d). RNA pol II and/or III activities were also detected in most nuclei isolated from embryos treated with 0.5 µg/ml of actinomycin D (Fig. 8 g). In contrast, onset of RNA pol I transcription was abolished (Fig. 6 f).

Thus, the treatment of whole embryos by low doses of actinomycin D provides a means to follow the changes in the distribution of the rRNA processing components in embryos without activation of RNA pol I transcription. From the same group of actinomycin D–treated (0.5 µg/ml) embryos, the nuclei were isolated 9 h after fertilization. Fibrillarin was already recruited to the nucleolar domain at this time (Fig. 7 e). Maternally derived pre-rRNAs were also detected by FISH in the nucleolar domain (Fig. 7 d). Indeed, formation of the nucleolar domain was initiated normally at the MBT, even if the onset of RNA pol I transcription was blocked. Based on these results, it appears that neither zygotic RNA pol I transcription nor the catalytic enzyme RNA polymerase I itself, are responsible for the recruitment of processing machinery.

Recruitment of the rRNA Processing Machinery Does Not Occur in the Absence of Maternal Pre-rRNAs in the Nucleolar Domain

As maternally derived pre-rRNAs were the only pre-rRNAs in embryonic nuclei at MBT, we wondered if they could play a role in recruiting the rRNA processing machinery to the nucleolar domain. A correlation could be established between the presence of pre-rRNAs in the nucleolar domain and the ability of the rRNA processing machinery to be recruited to this domain. Two types of nuclei were experimentally induced in which pre-rRNAs were not detected by FISH, and in both cases the rRNA processing machinery was maintained in large PNBs. The first type of nuclei was isolated from embryos treated with 2 µg/ml of actinomycin D and collected 9 h after fertilization (Fig. 7, g–i). At this time, Br-UTP was incorporated into large foci (Fig. 8 a) not colocalized with fibrillarin (Fig. 8 a, b arrows). These foci were not further detected, probably due to changes in the transcription pattern during development. Transcription observed 9 h after fertilization was inhibited by α-amanitin, indicating that it corresponds to RNA pol II and/or III activities (Fig. 8 d). RNA pol II and/or III activities were also detected in most nuclei isolated from embryos treated with 0.5 µg/ml of actinomycin D (Fig. 8 g). In contrast, the onset of RNA pol II and III activities was inhibited in 86% of nuclei isolated from embryos treated with 2 µg/ml of actinomycin D (Fig. 8 j). In the latter nuclei, fibrillarin was maintained in large PNBs (Fig. 7 h and 8 k) and no pre-rRNA was detected by in situ hybridization (Fig. 7 g). In a few nuclei only, fibrillarin and pre-rRNAs were regrouped in the nucleolar domain (Fig. 7, g and h, arrowheads). These nuclei most likely escaped inhibition of RNA pol II and III activities by actinomycin D, as indicated by the detection of fibrillarin clustered to nucleolar domain in the few nuclei in which RNA pol II and III ac-
activities were detected (data not shown). The PNBs that remained in the transcriptionally quiescent nuclei (Figs. 7 h and 8 k) were larger than the PNBs I observed in nuclei 6 h after fertilization (Fig. 1 b). Nucleolin, U3, and U8 were also found in these large structures (Fig. 9, d and e). B23/NO38 was maintained in small diffuse PNBs similar to those observed in nuclei isolated 6 h after fertilization (data not shown). A second type of nuclei were in in vitro reconstituted sperm nuclei. Again, the absence of nucleolar domain formation was observed (Fig. 9, g–i). This is consistent with the transcriptional inactive state of these nuclei, which was previously reported (Bell et al., 1992).

Discussion

During early X. laevis embryogenesis, components of the rRNA processing machinery are first associated in bodies,

Figure 6. Fibrillarin is targeted to the nucleolar domain even in the absence of zygotic RNA pol I transcription. The fluorescent in situ transcription assay by immunolabeling of incorporated Br-UTP coupled to immunolabeling of fibrillarin was performed on nuclei isolated 12 h after fertilization from control embryos (A) and embryos treated with 0.5 μg/ml of actinomycin D (B). A, Sites of transcription were labeled in nuclei isolated from control embryos (a) and colocalized with fibrillarin in the nucleolar domain (b). This transcription was RNA pol I transcription because it was not inhibited by incubation of nuclei in 100 μg/ml of α-amanitin before and during incorporation of Br-UTP (c). B, When whole embryos were treated with 0.5 μg/ml of actinomycin D during development, no RNA pol I transcription was detected (e). Although RNA pol I activity was inhibited, fibrillarin was clustered in the nucleolar domain (f) as in control nuclei. Bars, 10 μm.

Figure 7. Presence of maternally derived pre-rRNAs for the recruitment of fibrillarin to the nucleolar domain. In situ hybridization of rRNA was performed after immunolabeling of fibrillarin on nuclei isolated 9 h after fertilization from control embryos (a–c) and embryos treated with 0.5 μg/ml (inhibition of RNA pol I; d–f) or 2 μg/ml of actinomycin D (inhibition of RNA pol I, II, and III; g–i). The nucleolar domain was formed in control and 0.5 μg/ml actinomycin D–treated nuclei. rRNAs (a and d) were colocalized with fibrillarin (b and e) in this domain. rRNA was not detected in most nuclei from embryos treated with 2 μg/ml of actinomycin D (g). In this case, fibrillarin was not recruited to the nucleolar domain, but was maintained in large PNBs in the nucleoplasm (h). Only a few nuclei formed a nucleolar domain containing rRNA and fibrillarin (arrowheads in g and h, respectively). The corresponding DNA staining with DAPI is shown (c, f, and i). Bars, 10 μm.
known as the PNBs, which are dispersed in the nucleoplasm. These components are subsequently recruited to rDNA loci to form the nucleolar domain (Fig. 10). Both of these steps play an important part of the nucleolar assembly process. Interestingly, these phenomena also occur during nucleolar assembly at the end of mitosis (Hadjiolov, 1985; Scheer and Benavente, 1990; Thiry and Gømessens, 1996). A comparison of these biological situations reveals the general features necessary for recruitment of the rRNA processing machinery.

**PNB Assembly as the First Step of Nucleologenesis**

In cultured cells, PNBs are formed at the end of mitosis when components of the rRNA processing machinery associate (Ochs et al., 1985a; Schmidt-Zachmann et al., 1987; Jiménez-Garcia et al., 1989; Auzum-Gélade et al., 1994). PNB-like structures with a similar composition also form in vitro in reconstituted interphasic nuclei (Bauer et al., 1994; Bell et al., 1992; Bell and Scheer, 1997; Bauer and Gall, 1997). During X. laevis embryogenesis, PNBs assemble in early blastula embryonic nuclei and remain as discrete structures throughout interphase (Verheggen et al., 1998). In this study, we demonstrate the existence of two types of PNBs in both X. laevis embryonic nuclei and permeabilized sperm nuclei reconstituted in egg extracts. The components in PNBs I are all involved in early steps of rRNA processing, whereas the components found in PNBs II are involved in a late step of processing. Distinct PNBs for proteins involved in early and late steps of rRNA processing were also described in telophase cells (Fomproix and Hernández-Verdun, 1999; Savino et al., 1999).

To date, the mechanisms that govern PNB assembly are poorly understood. By systematic depletion of the egg extract, it has been shown that fibrillarin, nucleolin, B23/NO38, and U3 are dispensable for PNB assembly in reconstituted nuclei (Bell and Scheer, 1997). At the end of mitosis, PNB formation and the onset of transcription occur simultaneously, suggesting a common regulatory event (Fomproix et al., 1998). Nevertheless, PNB assembly occurs in the absence of transcription in both cultured cells (Morcillo et al., 1976; Benavente et al., 1987) and reconstituted nuclei (Bell et al., 1992). Nucleolus-like particles with features of PNBs were assembled in a soluble extract of nucleoli (Trimbur and Walsh, 1993). PNBs were also induced in interphasic cells which had been released from hypotonic
nuclei isolated from embryos treated with 2 μg/ml actinomycin D. The nucleolar domain was not formed in most control embryos. This domain appeared dense by phase-contrast microscopy (c). The nucleolar domain in nuclei isolated 9 h after fertilization from experiment, nucleolin (a) and fibrillarin (b) were colocalized in or in vitro reconstituted nuclei. In a double immunolabeling experiment, nucleolin (a) and fibrillarin (b) were colocalized in the nucleolar domain in nuclei isolated 9 h after fertilization from control embryos. This domain appeared dense by phase-contrast microscopy (c). The nucleolar domain was not formed in most nuclei isolated from embryos treated with 2 μg/ml of actinomycin D. Inhibition of RNA pol I, II, and III). Instead, nucleolin (d) and fibrillarin (e) were maintained in large PNBs. A similar distribution was also observed in vitro reconstituted nuclei (g and h). PNB structures, maintained at the MBT stage in treated embryos, were easily distinguished in nuclei by phase-contrast microscopy (f) and similar to structures formed in vitro reconstituted nuclei (i). Bars, 10 μm.

Figure 9. Fibrillarin and nucleolin were maintained in large PNBs throughout development in the presence of actinomycin D or in vitro reconstituted nuclei. In a double immunolabeling experiment, nucleolin (a) and fibrillarin (b) were colocalized in the nucleolar domain in nuclei isolated 9 h after fertilization from control embryos. This domain appeared dense by phase-contrast microscopy (c). The nucleolar domain was not formed in most nuclei isolated from embryos treated with 2 μg/ml of actinomycin D. Inhibition of RNA pol I, II, and III). Instead, nucleolin (d) and fibrillarin (e) were maintained in large PNBs. A similar distribution was also observed in vitro reconstituted nuclei (g and h). PNB structures, maintained at the MBT stage in treated embryos, were easily distinguished in nuclei by phase-contrast microscopy (f) and similar to structures formed in vitro reconstituted nuclei (i). Bars, 10 μm.

shock and transferred to normal isotonic medium, even in the presence of actinomycin D (Zatsepina et al., 1997). During X. laevis embryogenesis, PNBs are assembled from maternally derived components at a transcriptionally silent stage (Verheggen et al., 1998). In the present study, we observe PNBs in early and late stage nuclei from embryos treated with high concentrations of actinomycin D, confirming that PNB assembly during X. laevis embryogenesis is totally independent of any transcription.

Transcription and Processing Machineries Assemble in Bodies at Locations Distinct from RNA Synthesis and Processing Sites

A accumulation of machineries in domains distinct from their effector sites is not limited to rRNA processing machinery. We also showed that RNA pol I was not at rDNA loci at the time of nucleolar domain formation during X. laevis embryogenesis. Several extranucleolar foci containing RNA pol I distinct from PNBs were observed in both blastula nuclei (Bell and Scheer, 1999) and in vitro reconstituted nuclei (Bell et al., 1997). TBP is known to accumulate in these foci at the gastrula stage (Bell and Scheer, 1999). However, the significance of the presence of these complexes in nucleoplasmic foci remains to be elucidated.

Several examples of domains for accumulation of transcription and processing machineries have been reported. Stress granules, containing the transcription factor HSF1, formed in response to heat shock and independent of the sites of active transcription (Jolly et al., 1999a). Speckled distribution has been described for components of the mRNA processing machinery (for reviews see Moen et al., 1995; Huang and Spector, 1996a; Lamond and Earnshaw, 1998; Misteli and Spector, 1998). Speckles are prominent in cells with low transcriptional activity, whereas upon activation of transcription, factors are recruited from speckles to sites of active transcription (Jiménez-García and Spector, 1993; Huang and Spector, 1996b; Jolly et al., 1999b). In addition, coiled bodies are sites where components of the processing machinery localize, but the precise role of these bodies is not yet defined (Boudonck et al., 1999; Frey et al., 1999). Recently, a model was proposed in which the coiled body (cajal body) is the site of preassembly of RNA transcription and processing complexes before transport to the appropriate genes (Gall et al., 1999).

PNB Recruitment to the Nucleolar Domain as the Second Step of Nucleologenesis

Fusion of the PNB components to the nucleolar domain constitutes the second event of delivery of the processing machinery to the nucleolus. This stage was shown to be dependent on RNA pol I activity in cultured cells at the end of mitosis (Jiménez-Martin et al., 1974; Ochs et al., 1985a; Benavente et al., 1987; Scheer and Benavente, 1990). In contrast, we showed that during the de novo assembly of the nucleolus in X. laevis embryos, neither RNA pol I activity, nor the catalytic enzyme RNA pol I itself were required for this recruitment process. Formation of the nucleolar domain in the absence of RNA pol I has been observed in yeast mutant strains in which rRNA was transcribed from plasmids containing rDNA flanked by an RNA pol II-dependent promoter (Oakes et al., 1998). However, the authors did not exclude the possibility that a transcription factor, rather than the RNA pol I itself, was involved (Oakes et al., 1998).

Recruitment of the rRNA processing machinery has been shown to be mediated by the COOH-terminal domain (CTD) of the RNA pol II (Corden and Patturajan, 1997; McCracken et al., 1997; Steinmetz, 1997). Even in the presence of pre-rRNA in the transcription sites, the processing machinery is not recruited when the RNA pol II CTD is deleted (Misteli and Spector, 1999). A different recruiting mechanism is used for rRNA processing machinery as RNA pol I is dispensable for nucleolar domain assembly during X. laevis embryogenesis.

Protein recruitment to a specific location can be controlled by reversible phosphorylation, which could provide an attractive mechanism for recruitment of PNB to the nucleolar domain. For example, the nuclear import of nucleolin in early blastula of X. laevis has been shown to depend on its phosphorylation (Mebmer and Dreyer, 1993). The phosphorylation state also influences the subnuclear distribution of SR (Ser/Arg-rich) proteins (Misteli and Spector, 1998) and their activity in splicing of mRNA during
A hypothesis could be that recruitment of the rRNA processing machinery to the nucleolar domain involves changes in phosphorylation of proteins during X. laevis embryogenesis. The major event for recruitment of the rRNA processing machinery is likely to be the presence of pre-rRNAs in the nucleolar domain. In cultured cells, the onset of rDNA transcription at the end of mitosis could provide pre-rRNAs required for recruitment of the rRNA processing machinery to the nucleolar domain (Scheer et al., 1993). This re-assembly of PNBs is prevented if activation of rDNA transcription is inhibited (Benavente et al., 1987; Scheer and Benavente, 1990). Remarkably, during X. laevis embryogenesis, we observed that most of B23/NO 38 was recruited later than fibrillarin and nucleolin. A comparable situation was observed for nucleolus formation in telophase cell (Fomproix and Hernandez-Verdun, 1999; Savino et al., 1999). Considering that steps for nucleolar assembly are common to both embryonic and cultured cells at the end of mitosis, it is tempting to speculate that nucleolar assembly involves common regulatory pathways in both cases. The fact that components of rRNA processing machinery involved in distinct steps of processing are delayed in their recruitment could be in itself a regulatory mechanism for their stepwise involvement in rRNA processing. This is raising the question whether alteration of the timing of the recruitment process might impair processing of rRNA.
The authors are grateful to M. Schmidt-Zachmann for providing anti-pol I and anti-23S antibodies and to D. Roche for preparing the X laevis eggs and embryos. We thank P. Ridgway, R. Bastos, and A.-L. Henni for critical reading of the manuscript.

This work was supported in part by grants from the Centre National de la Recherche Scientifique (programme Biologie Cellulaire no. 96098) and l’A association pour la Recherche sur le Cancer (contracts nos. 9143 and 5304 to D. Hernandez-Verdun, and no. 1030 to G. A.imumi). C. V. erhegen was a recipient of a fellowship from l’A association pour la Recherche sur le Cancer.

Submitted: 20 September 1999
R evised: 29 February 2000
A ccepted: 7 M arch 2000

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