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

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**Abstract.** Transcription and splicing of messenger RNAs 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, 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 • *X enopus laevis* development

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**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) trans-* segmentation* 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 involves cleavage, methylation, and pseudouridylation of the primary rRNA (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 Aen 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.

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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; mR Na, 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 Weisenberger, 1994; B even et al., 1996; D undl et al., 1997; Zatsespin et al., 1997). Fibrillarin, nucleolin, Nop52, PM-Scl 100/exosome, and protein B23 are found within these PNBs, as are the U 3 and U 14 snoRNA s (A zum-Gélade et al., 1994; G autier et al., 1994; Jiménez-Garcia et al., 1994; B even et al., 1996; M itchell et al., 1997; Fomproix and H ernández-Verdun, 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 Weisenberger, 1994). Distinct PNBs are involved in the delivery of specific processing complexes to the nucleolar domain (Fomproix and H ernández-Verdun, 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.

The PNBs fuse at the nucleolar organizer regions (NORs) at the end of mitosis, when rRNA synthesis resumes (Jiménez-Garcia et al., 1994; Fomproix et al., 1998). This fusion is inhibited by microinjection into mitotic cells of antibodies directed against RNA polymerase I (RNA pol I; Benavente et al., 1996; Mitchell et al., 1997; Fomproix and Hernández-Verdun, 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 RNA pol I—dependent transcription (V erhogen 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 (B rown and Littna, 1964; Newport and Kirschner, 1982). At the midblastula transition (M BT), RNA pol II— and III—dependent transcription is activated, whereas RNA pol I transcription is initiated later (Shiokawa et al., 1981a,b; 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 M BT, scattered PNBs containing fibrillarin exhibit similar ultrastructural features to postmitotic PNBs and at M BT fibrillarin regroups around the rDNA with maternal pre-rRNA s (V erhogen et al., 1998). At M BT, the association of rDNA s with UBF was demonstrated (B ell et al., 1997; V erhogen 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 M BT 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, Nopp180, protein B23 (NO 38 in X. laevis), U 3, and U 8 (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 I 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

Antibodies with the following specificities were used: a human autoimmune serum directed against fibrillarin (Gautier et al., 1994); a rabbit polyclonal serum directed against human fibrillarin (a kind gift of C. Faucher, LMBE, 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 pBR 322 (clone pX c7, kindly provided by F. A maldi, Université T o R Vergata, R oma, Italy). Double-stranded DNA probes were labeled with the nick-translation kit (GIBCO BRL) according to the manufacturer’s instructions using biotin-14-dCTP. Probes for the detection of U 3 and U 8 are oligonucleotides complementary to three regions (positions 9-31; 63-85; and 101-122 for U 3; and 33-60; 72-93; and 107-136 for U 8). They were labeled with biotin-14-dCTP using 3' terminal transferase (Boehringer Mannheim).

Assembly of Nuclei in X. laevis Egg Extract

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. Demembranated X. laevis sperm nuclei were prepared and permeabilized with lysosomectin (A Imouzi, 1998). For nuclear assembly, 105 sperm heads were added to 100 μl of egg extract and maintained at 23°C. After 45 min, when maximal decondensation of the nuclei occurred, nuclei were processed for photonic and electron microscopies.

Preparation of Embryonic Nuclei from X. laevis

Embryos were produced by in vitro fertilization (A Imouzi and W olffe, 1995) and allowed to develop at 23°C in 0.1× modified Barth solution (G urdon and W ickens, 1983) for different times after fertilization. At this temperature, embryos were collected at the following stages: early blastula, 6 h after fertilization (stage 8); midblastula, 7 h after fertilization (stage 8.5); late blastula, 9 h after fertilization (stage 9); early gastrula, 10 h after fertilization (stage 10); gastrula, 11 h after fertilization (stage 10.5); and late gastrula, 13 h after fertilization (stage 12); as specified by Nieuwkoop and Faber (1994). In some cases, actinomycin D was added to inhibit the onset of transcription during development. A dissociation of the
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 \textit{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 \textit{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 \textit{X. laevis}, were also detected in PNBs I by FISH (see Fig. 3). On the contrary, B23/NO38, a nucleolar protein involved in a late step 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; Baur 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 as in embryonic nuclei. Permeabilized sperm nuclei incubated in a low-speed 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 E M, 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 \( \mu \) m) 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 \( \mu \) m) were gray without visible dense granules (Fig. 2 c). Based on their size, distribution

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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% OSO\(_4\) 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 (BrUTP) incorporation into embryonic nuclei was performed as previously described (Verheggen et al., 1998). In brief, nonfixed nuclei were permeabilized and incubated in transcription buffer in the presence of BrUTP for 20 min at room temperature (Masson et al., 1996). Before immunolabeling, the nuclei were fixed with 2% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100. A monoclonal anti-BP-deoxouridine antibody which also recognizes BrUTP (Boehringer Mannheim) was used. Human antifibrillarin antibodies were used as a nucleolar marker. Transcription and fibrillarin signals were obtained using FITC-conjugated goat anti–mouse and TRITC-conjugated goat anti–human antibodies (Jackson ImmunoResearch Laboratories, Inc.) and mounted with an anti–fading reagent (Citrifluor).

In situ hybridization of rRNAs was performed after immunolabeling as previously described (Verheggen et al., 1998). The rRNA hybridization mixture contained 40% formamide (GIBCO BRL), 10% (wt/vol) dextran sulfate (Sigma Chemical Co.), 50 ng/\( \mu \)l sonicated salmon testes DNA (Sigma Chemical Co.), and the biotinylated rRNA probe diluted to a final concentration of 1 ng/\( \mu \)l in 2× SSC. For the detection of U3 and U8, the hybridization mixture contained 30% formamide, 10% (wt/vol) dextran sulfate, 50 ng/\( \mu \)l sonicated salmon testes DNA, and the 3′ end biotinylated oligonucleotides complementary to U3 and U8 diluted to a final concentration of 2 ng/\( \mu \)l in 2× SSC. A secondary control for the detection of RNA, hybridization was preceded by RNase digestion as described (Highett et al., 1993).

Optical Microscopy

Images were taken with a Leica epifluorescence microscope equipped with a thermoelectronically cooled charge-coupled device (CCD) camera (Leica). Grayscale images were collected separately with filter sets for FITC and rhodamine/TRITC using an oil immersion lens (63×, NA 1.4 plan A porchomat). Grayscale images were pseudocolored and merged using the Adobe Photoshop 5.0 software.

Electronic 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% OSO\(_4\) 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 EM 412 electron microscope.

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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 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 Ca^{2+}- and Mg^{2+}-free medium, conditions that dissociate...
its sensitivity to and actinomycin D–treated embryos, was characterized by Br-UTP incorporation in isolated nuclei from control condition (see Materials and Methods). Transcription, revealed by Br-UTP incorporation in isolated nuclei from control condition favoring the detection of 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 to block cell division. Nuclei, isolated from dissociated embryos while they remain within the vitellin membrane, were comparable in size to nuclei from control embryos that were developed in 0.2 and 10 μg/ml of actinomycin D. When the nucleolar domain began to assemble in nuclei 7 h after fertilization, maternally derived rRNAs were detected (b). No RNA polymerase I was seen colocalized to the FISH signal at this time (a; arrowheads). Instead, RNA polymerase I appeared as speckles dispersed in the nucleoplasm. This distribution was maintained in nuclei 9 h after fertilization (d). RNA polymerase I was not yet colocalized with rRNA (e). Only after 11 h of development, RNA polymerase I (g) was seen partially colocalized with rRNAs (h). The corresponding DNA staining with DAPI is shown (c, f, and i). Bars, 10 μm.

Figure 5. RNA polymerase I is not associated with the newly formed nucleolar domain. Embryonic nuclei isolated 7, 9, and 11 h after fertilization were labeled with an antibody to RNA polymerase I (a, d, and g) before performing in situ hybridization of the rRNAs (b, e, and h). When the nucleolar domain began to assemble in nuclei 7 h after fertilization, maternally derived rRNAs were detected (b). No RNA polymerase I was seen colocalized to the FISH signal at this time (a; arrowheads). Instead, RNA polymerase I appeared as speckles dispersed in the nucleoplasm. This distribution was maintained in nuclei 9 h after fertilization (d). RNA polymerase I was not yet colocalized with rRNA (e). Only after 11 h of development, RNA polymerase I (g) was seen partially colocalized with rRNAs (h). The corresponding DNA staining with DAPI is shown (c, f, and i). Bars, 10 μm.

It was first necessary to check the ability of actinomycin D when applied to whole embryos to selectively block RNA pol I transcription. This prompted us to use embryos 12 h after fertilization and in situ transcription assays in conditions favoring the detection of RNA pol I transcription (see Materials and Methods). Transcription, revealed by Br-UTP incorporation in isolated nuclei from control and actinomycin D–treated embryos, was characterized by its sensitivity to α-amanitin. In control embryos, the transcription detected in nuclei isolated 12 h after fertilization (Fig 6 a) was RNA pol I–dependent (Fig 6 c). Indeed, transcription was not sensitive to 100 μg/ml of α-amanitin and consequently was not RNA pol II- and III–dependent. Accordingly, with RNA pol I transcription the transcripts were colocalized with fibrillarin (Fig. 6 b and d). In contrast, RNA pol I transcription was not detectable in 95% of the nuclei isolated from embryos treated with 0.5 μg/ml of actinomycin D and collected 12 h after fertilization (Fig. 6 e). This demonstrates inhibition of RNA pol I in actinomycin D–treated (0.5 μg/ml) whole embryos. Interestingly, the recruitment of fibrillarin occurred even when 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,
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 Goessens, 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; A zum-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 Hernandez-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

![Figure 8](image-url)
nuclei isolated from embryos treated with 2 μg/ml actinomycin D (c). The nucleolar domain was not formed in most control embryos. This domain appeared dense by phase-contrast microscopy (f) and similar to structures formed in in vitro reconstituted nuclei. In a double immunolabeling experiment, nucleolin (a) and fibrillarin (b) were colocalized in control embryos treated with 2 μg/ml 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 in vitro reconstituted sperm 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 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 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 in vitro reconstituted sperm 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 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.

**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-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).

**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).
development of *Ascaris lumbricoides* (Sanford and Bruzik, 1999). 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-rRNA s in the nucleolar domain. In cultured cells, the onset of rDNA transcription at the end of mitosis could provide pre-rRNA s 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.
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