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Original article

Non-isotopic detection of nucleolar transcription in pre-implantation mouse embryos

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Abstract – Nucleolar transcription was analysed in permeabilized pre-implantation mouse embryos at the four-cell, eight-cell, morula and early blastocyst stages using confocal microscopy to detect incorporated 5-bromouridine. The results demonstrated that the patterns of nucleolar transcription sites were common for all embryonic stages studied. They consisted most frequently of tightly associated groups of transcription foci similar to those encountered in somatic interphase cells. In addition, the nucleologenesis accompanying each cell cycle apparently gave rise to a different fluorescent pattern, that is to spatially separated fluorescent foci in the cells just after the resumption of rRNA synthesis. An immunoelectron microscopic analysis of the nucleolar transcription was also performed in the eight-cell embryos. A signal, usually consisting of clustered gold particles, was found specifically within nucleolar dense fibrillar components. This result was in agreement with established findings, which identify dense fibrillar component as the major site of nucleolar transcription in somatic cells. © Inra/Elsevier, Paris

mouse embryo / nucleolus / transcription site / immunodetection of incorporated bromouridine

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Résumé – Détection non-isotopique de la transcription nucléolaire dans les embryons pré-implantatoires de souris. La transcription nucléolaire a été analysée dans les embryons de souris au stade quatre cellules, huit cellules, morula et blastocyste précoce par microscopie confocale après incorporation de 5-bromo-uridine. Les résultats obtenus montrent que l'organisation de sites de transcription était commune à tous les stades étudiés. Ils sont groupés de manière similaire à ceux observés dans les cellules somatiques. De plus, la nucléologenèse accompagnant chaque cycle cellulaire donne lieu à un autre type de distribution, en sites isolés apparemment observés dans les cellules au moment de réinitiation de synthèse d'ARN nucléolaire. L'analyse immunocytochimique ultrastructurale des sites de transcription nucléolaire a été effectuée au stade de l'embryon à huit cellules. Le signal des particules d'or groupées était associé au composant nucléolaire dense. Ce résultat est en accord avec les données établies pour les cellules somatiques dans lesquelles le composant fibrillaire dense est considéré comme le site majeur de transcription nucléolaire. © Inra/Elsevier, Paris

embryon de souris / nucléole / site de transcription / immunodétection / incorporation de bromo-uridine

1. INTRODUCTION

The synthesis of mammalian ribosomal 18S, 5.8S and 28S rRNA occurs in a prominent nuclear domain called the nucleolus. All these rRNAs are transcribed as a single transcription unit by RNA polymerase I in the form of a 45S pre-rRNA which is processed to smaller RNA species. Corresponding genes are clustered in tandem repeats on a special chromosomal domain known as the nucleolar organizing region (NOR) [14, 26]. The number of NORs varies among species. In the mouse haploid genome, up to six chromosomes may contain a NOR region [6] bearing approximately 100 ribosomal genes in total [26]. Besides the transcription and processing of the 45S pre-rRNA, ribosome biogenesis occurs inside the nucleolus. It includes importing of the 5S rRNA, the snRNAs, and the nucleolar and ribosomal proteins, the formation of pre-ribosomal particles and their subsequent export to the cytoplasm. A large number of processes take place in the active nucleolus, but its ultrastructure only makes it possible to distinguish five structurally different components: fibrillar centres (FC), dense fibrillar components (DFC), granular components (GC), nucleolar

interstices and clumps of condensed chromatin [12, 36–38].

In somatic mammalian cells, the nucleolus breaks down and reforms during each cell cycle [33]. The nucleolus disintegrates during prophase and rRNA synthesis ceases. The nucleolus begins to be reformed in telophase during which pre-nucleolar bodies (PNBs) appear. They contain several nucleolar constituents and are scattered throughout the reforming daughter nuclei [1–3, 15, 21, 22, 30, 33]. When rRNA synthesis resumes, PNBs fuse around the NORs and subsequently only the GC appear at the periphery of the developing nucleolus [33, 35].

In addition to cell cycle nucleologenesis, a gradual maturation of nucleoli occurs during early embryonic development [9, 11, 24, 25]. In the case of the mouse zygote, the pronuclei do not possess transcriptionally active nucleoli. Instead, morphologically compact nucleolus precursor bodies (NPBs) are observed [9, 11]. Biochemical experiments have demonstrated that rRNA synthesis does not begin before the late two-cell stage in the mouse embryo [8, 23]. The periphery of the NPB changes at the same time and a new structure, the nucleonema, composed of ribonucleoproteins (RNPs), begins to emerge

from the NPBs [5, 16]. During further embryonic development, the NPBs gradually disappear and at the early blastocyst stage, the nucleolus becomes morphologically identical to the somatic one [11].

The first signs of the nucleolar synthesis have been autoradiographically detected at the four-cell stage [11, 16]. The intensity of autoradiographic labelling gradually increases during further embryonic development and is particularly high at the early blastocyst stage [11]. Ultrastructural observations have shown that [^3H]uridine is incorporated in the DFC after a 10-min pulse [11].

As an alternative to the autoradiographic approach, a non-isotopic immunocytochemical method of RNA synthesis detection can also be used [4, 7, 19, 20, 27, 32, 39]. It has much higher resolution and is based on the use of 5-bromouridine-5'-triphosphate (BrUTP) which is incorporated as 5-bromouridine in the RNA. The aim of this study was to provide a non-isotopic light microscopic description of nucleolar transcription in embryonic mouse cells from the four-cell to blastocyst stage and an electron microscopic mapping of the transcriptional sites inside the nucleolar structure in the eight-cell embryo.

2. MATERIALS AND METHODS

2.1. Embryo collection

The embryos were collected from 4-week-old superovulated C57BL/6J mice. The females were injected with 10 IU of pregnant mare serum gonadotropin (Sigma Co., USA) followed 46 h later with 5 IU of human chorionic gonadotropin (Sigma Co., USA), before being placed overnight in individual cages with males. The following morning, the effectiveness of mating was confirmed by the presence of a vaginal plug. Mouse embryos were collected at standard intervals after human chorionic gonadotropin treatment [17] in HEPES-

buffered M2 medium (Sigma Co., USA). Embryos were cultured shortly before BrUTP incorporation and/or fixation in M16 medium (Sigma Co., USA) containing 4 mg·mL⁻¹ of bovine serum albumine (BSA) [41].

2.2. BrUTP incorporation

The embryos (four-cell, eight-cell, morula and blastocyst stages) were washed three times in cold PBS (150 mM NaCl, 10 mM phosphate buffer, pH 7.4), then three times in cold D buffer (100 mM KCl, 50 mM TrisHCl, 5 mM MgCl₂, 0.5 mM EGTA, 1 mM PMSF, 1 mM DTT, 0.16 M sucrose, 10 % glycerol), permeabilized in D buffer containing 0.2 % Triton X-100 for 3 min on ice and washed three times in cold D buffer. Transcription was performed in D buffer supplemented with 25 mM S-adenosyl-L-methionine, 5 U·mL⁻¹ RNase inhibitor from human placenta (Boehringer Co., Mannheim, Germany), 0.2 mM ATP, GTP, CTP and BrUTP for 5 or 15 min at 32 °C [31]. Transcription was stopped by washing with cold PBS. In a control experiment, the transcription by RNA polymerase II was inhibited by α -amanitin which was present in the post-permeabilization washes and in the transcription buffer at concentration 12 $\mu\text{g}\cdot\text{mL}^{-1}$.

2.3. Immunofluorescence and confocal microscopy

After transcription and washes in PBS, the embryos were fixed in 2 % paraformaldehyde in PBS for 20 min, incubated with anti-bromodeoxyuridine antibody (Boehringer Co., Mannheim, Germany) for 150 min, washed in PBS, incubated with the secondary antibody conjugated with FITC or TRITC for 150 min, washed in PBS and mounted in Mowiol on microscopic slides. The DNA was stained with DAPI dye (1 $\mu\text{g}\cdot\text{mL}^{-1}$) during the secondary antibody incubation step. The embryos were examined with either an Olympus BH2 microscope, or a Bio-RAD MRC 1000 confocal microscope attached to a Nikon Diaphot inverted microscope (running under Comos software) using Kalman filtration. The set up of the confocal microscope did not, however, enable the visualization of the DAPI staining. In the control experiments, digestion by RNase-free DNase (100 $\mu\text{g}\cdot\text{mL}^{-1}$ in PBS with 5 mM

MgCl₂) or RNase A (400 µg·mL⁻¹ in PBS) was performed for 30 min at 37 °C before the antibody treatment.

2.4. Immunoelectron microscopy

After transcription and washes in PBS, the eight-cell embryos were fixed in 8 % paraformaldehyde in 0.2 M Pipes, pH 6.95 for 12 h, washed in PBS, dehydrated in ethanol and propyleneoxide and embedded in epon. Incorporated bromouridine was detected in ultrathin sections by means of anti-bromodeoxyuridine monoclonal antibody diluted in PBS containing 5 % fetal calf serum (FCS), washed three times in PBS, incubated with 6 nm gold-conjugated anti-mouse antibodies (goat anti-mouse IgG, Aurion, the Netherlands) diluted in PBS containing 5 % FCS, washed with PBS and water. Ultrathin sections stained with uranyl acetate were examined with Jeol 1200EX and Opton EM 109 electron microscopes.

2.5. Conventional electron microscopy

The eight-cell embryos were fixed for 60 min in 2.5 % glutaraldehyde and 0.75 % paraformaldehyde in a 0.06 M cacodylate buffer, pH 7.4 supplemented with 0.05 % potassium ferricyanide which provides higher contrast after osmification. After washing in this buffer at 4 °C, the embryos were postfixed in 1 % OsO₄ overnight, dehydrated in ethanol and propyleneoxide and embedded in Polybed (Polysciences, Inc., USA). Ultrathin sections contrasted with uranyl acetate and lead citrate were observed with a Jeol 1200EX electron microscope.

3. RESULTS

Conventional fluorescence microscopy was originally proposed for use in these kind of studies. However, it immediately appeared that this approach was not convenient owing to the size of embryos. Confocal microscopy has now become the standard tool. Nevertheless, conventional

fluorescence microscopy remains necessary for the identification of mitotic and early interphase cells as judged from DAPI staining (see below).

For preferential visualization of sites of nucleolar transcription we used the modified protocol enhancing nucleolar over extranucleolar transcription described in the Materials and methods. Under these conditions the extranucleolar signal was not detectable. Nevertheless, in order to exclude the possibility that RNA polymerase II contributed to the signal we analyzed embryos after an α -amanitin treatment. No changes in the signal pattern were seen. The signal was eliminated by RNase A, but not by DNase treatment (results not shown). Within the frame work of this study we identified the nucleolar transcription sites with sites of rDNA transcription.

The intensity of signal was higher for the 15-min incorporation period than for the 5-min one and so 15 min were used for most of the embryos investigated. Interestingly, the intensity of signal in the labelled nuclei was comparable for all the early embryos stages. The signal was also detectable when the 5-min interval was used.

The confocal microscopy results demonstrated that there were three patterns of nucleolar transcription sites, i.e. clustered transcription foci, spatially separated foci and cells lacking transcription signals. These patterns were seen in all the embryonic stages studied (see below).

The nucleolar signal consisted of tightly associated groups of synthetic foci in most cells, similar to the situation encountered in somatic interphase cells (*figure 1*). The number of foci usually ranged between 12 and 25. As the foci were clustered the individual foci could not always be spatially resolved and as a result these numbers are probably underestimated.

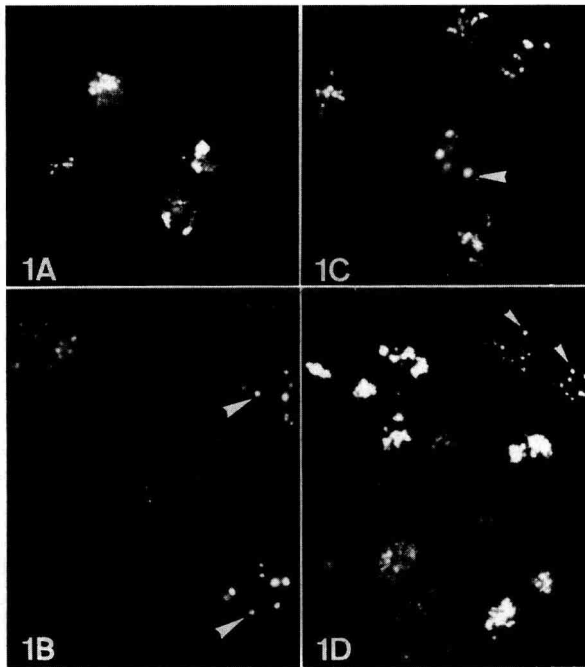


Figure 1. Selected examples of the localization of rRNA synthetic sites in embryonic cells by means of confocal microscopy. The fluorescence intensity in the figures has only relative meaning as explained in the Discussion. Embryos exhibiting transcription patterns such as separated foci, or cells lacking transcription signal, were infrequent: (A) four-cell embryo, (B) eight-cell embryo, (C) morula, (D) blastocyst. The nucleolar transcription sites are clustered in most cells. Separated foci designated by arrowheads are visible only in some cells (B, C, D). Only tightly clustered foci are seen in (A). At the morula and blastocyst stages, the cells exhibiting clustered foci prevailed. Only a few cells contained separated foci. Some cells at morula and blastocyst stages (C, D) apparently lack the nucleolar signal in the presented figure. The cells without a fluorescent signal are generally out of focus and a majority do in fact exhibit nucleolar fluorescence signal in neighboring optical sections except for mitotic phases. Original magnification (A) $\times 650$, (B) $\times 1\,250$, (C) $\times 1\,200$, (D) $\times 960$.

Two additional although much less frequent patterns of transcription sites were sometimes observed in some embryos. These were that the nucleolar transcriptional activity was not detected in some cells or that the signal was observed at spatially separated foci in some nuclei (*figure 1B–D*). If these patterns were observed, then the four-cell embryos exhibited a homogeneous signal as they

contained either cells with clustered foci in most cases (*figure 1A*) or, in rare cases, the embryos exhibited only cells with no signal and/or cells with separated foci. At later embryonic stages, particularly at the morula and blastocyst stages (*figure 1C, D*), the investigated embryos contained mainly cells with clustered foci, but some embryos contained also a variable, usually low number of cells exhibiting sepa-

rated foci or cells lacking the signal. Interestingly, these cells usually formed a small group of neighbouring cells within the embryo. The number of spatially separated foci was eight or less in all embryos investigated.

The results obtained from DAPI staining in conventional fluorescence microscopy showed that these additional transcription patterns were observed in

mitotic cells (cells without transcription signals) and in early interphase cells (cells with spatially separated transcription foci) (results not shown) apparently reflecting biosynthetic changes accompanying the nucleolar breakdown and reformation.

At the electron microscopy level, the signal was specifically found in the nucleolar DFC of the eight cell embryos (*figures 2 and 3*: a conventional ultrastruc-

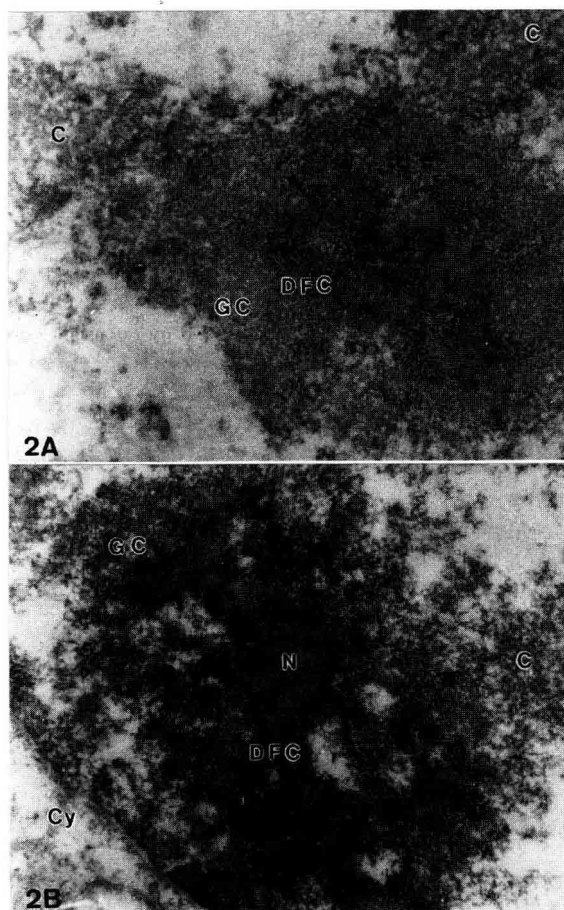


Figure 2. Ultrastructural immunolocalization of incorporated bromouridine (after 15 min of bromouridine incorporation) in the nucleolus of the eight cells embryo. The labelling (arrowheads) is specifically located in the nucleolar dense fibrillar components (DFC). Small groups of gold particles are often observed. NPB remnants (N) are devoid of gold particles. Cy = cytoplasm, C = chromatin, GC = granular components. Original magnification (A) $\times 50\,000$, (B) $\times 75\,000$.

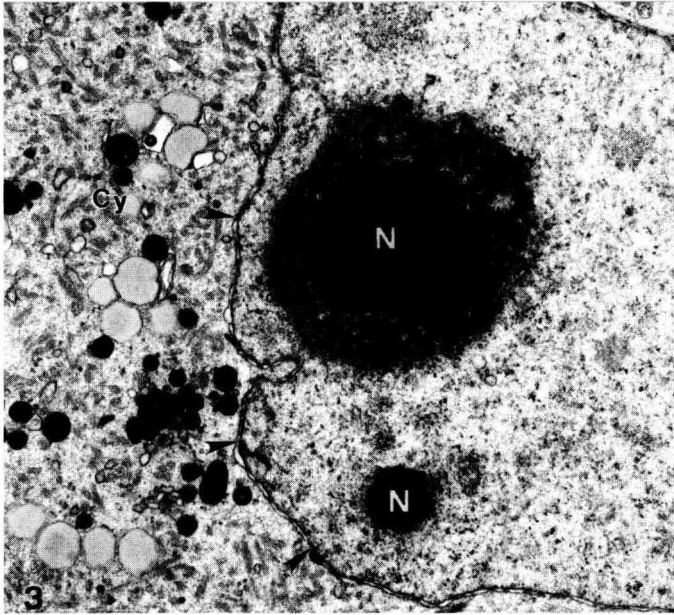


Figure 3. The cell nucleus of a eight-cell embryo embedded in Polybed. The nucleus contains two compact NPBs (N), one of which is already surrounded by nucleolar components typical of somatic nucleoli. Arrowheads designate the nuclear envelope, Cy = cytoplasm. Original magnification $\times 11\,000$.

tural picture of the nucleus exhibiting prominent PNBs). Few gold particles were found over the FC and GC and the interior of the NPBs was not labelled. The gold particles were frequently found clustered. In comparison to the 15-min period for BrUTP incorporation, the signal observed in DFC was weaker for the 5-min incorporation interval (results not shown).

4. DISCUSSION

In this study we analysed nucleolar transcriptional activity in permeabilized pre-implantation embryos at the four-cell, eight-cell, morula and early blastocyst stages using confocal microscope detection of incorporated bromouridine. The results showed that there were no sub-

stantial differences in the organization of nucleolar transcription sites among the different pre-implantation mouse embryo stages except the four-cell stage. In most cells representing true interphasic cells, the transcription sites corresponded to clustered foci and were similar to the situation encountered in somatic cells [19, 20, 31, 39]. About 200 ribosomal genes are present in mouse diploid cells [26]. The large number of foci seen in most embryonic cells likely corresponded to the dispersion of ribosomal genes during interphase, as has been documented for vertebrate cells [13, 29]. The different labelling pattern observed in a few cells may be related to the nucleolar breakdown and reformation accompanying each mitosis. As the lack of nucleolar signal apparently reflected the cessation of rRNA synthesis

in mitotic cells, we suggest that the separated foci corresponded to the sites of early activation of ribosomal genes after resumption of rRNA synthesis. This interpretation was in agreement with the DAPI staining to identify mitotic and early interphase cells, respectively. The number of separated foci was in agreement with the total number of NORs as eight NORs were found in this strain of mice [6]. We therefore tentatively identified these foci with individual NORs. If true, such organization of rDNA transcription just after the resumption of rRNA synthesis seems to be a general phenomenon since a similar arrangement of transcription sites was observed in Hela cells as well [32]. The higher homogeneity in the fluorescence pattern seen in the four-cell embryos likely corresponded to a higher level of cell synchrony at this stage.

The ultrastructural mapping of nucleolar transcription sites in the eight-cell embryo was in agreement with a previous autoradiographic study identifying the DFC as [^3H] uridine incorporation sites [11]. The signal frequently consisting of clustered gold particles was specifically located in nucleolar DFC. The results of the present study agreed with findings established for mature mammalian cells which identify the DFC as major sites of nucleolar transcription [31].

One may argue that the permeabilization of cells and the incubation conditions were too artificial and that the results so obtained do not reflect the natural situation in intact cells. We believe that the method used depicts genuine synthetic sites in early mouse embryos as it has already been proven to be a valuable tool in the study of somatic animal and plant cells as well as oocytes [7, 10, 18–20, 27, 28, 31, 39]. In addition, the present study was, at least at a qualitative level, in a complete agreement with previous autoradiographic results [11]. However, we emphasize that the early mouse embryo

exhibits specific features apparently due to its size. The nucleolar labelling was weaker in comparison to previous results obtained with cultured somatic cells (e.g. [31]). In order to have comparable signals with those obtained in established cultured cell lines which incorporated BrUTP during 5-min intervals, 10–15-min intervals should be used with early embryos. Also, a gradual increase in amount of labelling was observed between the four-cell embryo and blastocyst in the autoradiographic study [11], whereas the fluorescent signal intensity did not give clear quantitative results. The reason was that we had access to the confocal microscope at different times with consequently different adjustments so that the intensity levels obtained were unrelated. It is also possible that the BrUTP method does not reflect the processing of synthesized rRNA properly due, perhaps, to the interfering role of incorporated bromouridine ([7]; see also [34, 40] for mRNA).

In summary, we have shown that the non-isotopic BrUTP method is sensitive, rapid and has higher resolution than autoradiography, although it is perhaps not as quantitative for the detection of nucleolar synthesis in pre-implantation embryos at the light (confocal) and electron microscope levels. This technique could be useful for the study of the onset of nucleolar RNA synthesis in embryos of other mammalian species.

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NOTE ADDED IN PROOF

We have performed meanwhile a study on two-cell embryos in which we have not observed nucleolar transcription signal. Even though a possibility exists that the signal was too weak to be detected because of the experimental approach used, these results indicate that the onset of rRNA synthesis begins at four-stage mouse embryo.

ABBREVIATIONS USED IN THIS PAPER

NOR, nucleolar organizing region; FC, fibrillar center; DFC, dense fibrillar components; GC, granular components; PNB, prenucleolar body; NPB, nucleolus precursor body; BrUTP, 5-bromouridine-5'-triphosphate; BSA, bovine serum albumine; FCS, fetal calf serum.

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