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Endogenous Transcription Occurs at the 1-Cell Stage in the Mouse Embryo

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In most animal species, the earliest stages of embryogenesis are regulated by maternally inherited components, at least until the activation of the zygotic genome. Although the first proteins derived from the activation of the embryonic genome have been detected long ago at the 2-cell stage in the mouse, the exact timing of transcriptional activity resumption after fertilization is still a matter of debate. Any new information about this critical event is relevant to the practice of transgenesis and cloning of embryos as well as to the general understanding of the regulation of nuclear processes following fertilization. Using a new fluorescent method allowing the detection of *in vivo* RNA synthesis, we show that endogenous transcription by RNA polymerase II takes place unambiguously as early as at the late 1-cell stage. Furthermore, we demonstrate that transcription is first initiated in the paternal pronucleus.

INTRODUCTION

In most species, embryonic development begins by a transcriptionally silent period exclusively controlled by maternally inherited components. The period of the transition from maternal to embryonic control of development differs largely between species [1]. In the mouse, the first biochemical evidence of gene transcription is available at the 2-cell stage. Indeed, α-amanitin-sensitive expression of zygotic genes is first detected in 2-cell embryos [2] by the appearance of both stage-specific and common cellular proteins in two successive waves: a first *de novo* synthesis of a small set of proteins occurs at the early 2-cell stage [3, 4], while a second transcriptional burst during the late 2-cell stage results in a major transition in the pattern of polypeptide synthesis [2, 5]. Moreover, α-amanitin treatment of 1-cell embryos does not affect their development up to the late 2-cell stage [2, 6]. In addition, synthesis of paternally derived proteins becomes detectable from the 2-cell stage on [7]. From all these results, it has long been assumed that 1-cell embryos were transcriptionally inert.

However, some experiments suggest that transcription may already be initiated prior to the 2-cell stage in the mouse embryo. (i) A low level of internal incorporation of [^{3}H]adenosine into heterogeneous nuclear poly(A)$^-$ RNA was detected in the mouse pronuclei [8, 9]. (ii) Nuclear transfer experiments have shown that the cytoplasm of enucleated late 1-cell embryo supports the transcriptional activity of an α-amanitin-treated 2-cell-stage nucleus [10]. (iii) Transient expression of a reporter gene microinjected in the male pronucleus has been detected at the late 1-cell stage, at a relatively low level [11]. (iv) Analysis of subtracted cDNA libraries from preimplantation embryos showed that at least one gene exhibits a transient increase of its expression at the 1-cell stage [12].

Despite these studies, the occurrence and the exact timing of endogenous gene transcription in the mouse embryos at the 1-cell stage remained a matter of debate. To address this problem, we decided to test on intact mouse embryos a very sensitive method, recently used for the detection of RNA synthesis sites in somatic cells [13–15]. It is based on the immunofluorescent detection of bromouridine (BrU) incorporation into nascent RNA transcripts, using 5-bromouridine-5′-triphosphate (BrUTP) as precursor. We report here the successfull incorporation of BrUTP by endogenous RNA polymerase II in mouse embryos as early as at the late 1-cell stage.

MATERIALS AND METHODS

Collection and in vitro culture of embryos and BrUTP microinjections. Female C57/CBA mice (4–8 weeks old) were superovulated with intraperitoneal injection of 5 IU of pregnant mare's serum gonadotropin (PMSG; Folligon, Intervet) followed 48 h later with 5 IU of human chorionic gonadotropin (hCG; Chorulon, Intervet). They were then mated with C57/CBA males. Under these conditions, fertilization is assumed to occur approximately 12 h post-hCG injection

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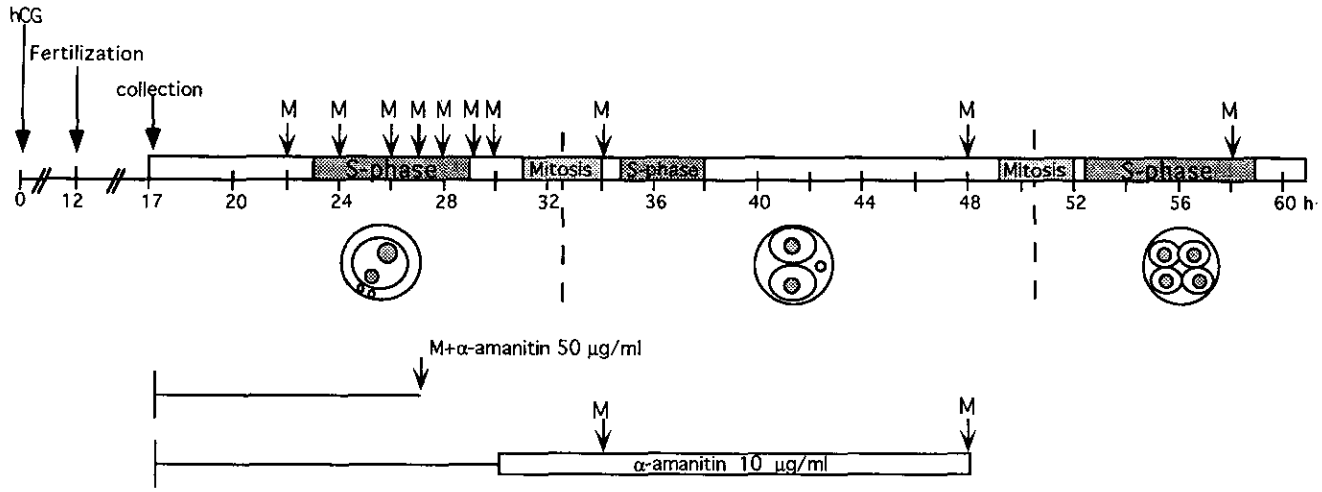

FIG. 1. Timing of mouse embryonic development and schedule of experimental variants. hCG injection is taken as reference for the approximate timing of mouse embryonic development. Microinjections (M) are indicated by arrows. Experimental protocols using drug treatment are represented by white boxes below the time line.

(hphCG) [16]. Fertilized eggs were recovered from ampullae at 17 hphCG, briefly treated with hyaluronidase (1 mg/ml), and further cultured in Whitten's medium [17] at 37°C in a humidified atmosphere of 5% CO_2 in air until the time of microinjection.

Knowing that the first two embryonic cell cycles each last approximately 20 h [18], we used the following temporal correspondence of mouse embryonic development: 22 hphCG and 26–29 hphCG, respectively, to early and late 1-cell stages, 34 hphCG and 48 hphCG to early and late 2-cell stages, and approximately 58 hphCG to the 4-cell stage (Fig. 1).

Embryos at these various development stages were microinjected in the cytoplasm with 100 m*M* BrUTP in 2 m*M* Pipes, pH 7.4, 140 m*M* KCl. The injected volume was 1–5% of the cell volume. After further culture for 15 or 60 min, embryos were fixed and then processed for *in situ* indirect immunofluorescence.

To block RNA polymerase II-dependent transcription, α-amanitin was used. Because of its low rate of uptake, two different procedures were performed depending on the cell stage to be studied. When probing the 2-cell stage, α-amanitin was added to the culture medium (10 μg/ml) at 28 hphCG, while in the case of the 1-cell stage, it was included in the injection buffer at 50 μg/ml. In the later case, its final intracellular concentration was estimated to be 0.5–2.5 μg/ml assuming that α-amanitin diffuses in the whole cell volume.

Figure 1 summarizes the schedule of the different experimental variants.

Fixation and immunofluorescence microscopy. Embryos were fixed in 2% (w/v) paraformaldehyde in phosphate-buffered saline (PBS; pH 7.35) for 20 min and then permeabilized in PBS containing 0.2% Triton X-100 for 15 min and blocked in PBS with 2% bovine serum albumin (BSA) for at least 1 h. Incubations with the first antibody (monoclonal mouse (IgG) anti-BrdU, Caltag Laboratories, 1:500 in PBS–2% BSA) and second antibody (fluorescein-conjugated goat anti-mouse IgG, Nordic Immunological Laboratories, 1:400 in PBS–2% BSA) were performed at room temperature for at least 3 and 1 h, respectively. Each step was followed by thorough rinsing in PBS for 20 min, the last rinse containing 2 μg/ml of the DNA-specific dye, Hoechst 33342. Embryos were then postfixed in 2% paraformaldehyde in PBS for 25 min before being observed, either *in toto* in small droplets of PBS, or after deposition on polylysine-coated slides mounted in citifluor (Citifluor Company). In control experiments, digestion by RNase-free DNase I (100 μg/ml in PBS, 5 m*M* $MgCl_2$) or RNase A (400 μg/ml in PBS) was performed for 30 min at 37°C just after the permeabilization step. Observations were performed on a Zeiss inverted microscope (Axiovet 35), equipped for epifluorescence microscopy. Images were captured with an intensified video camera (type 4336, Lhesa Electronique) and processed through a digital system (Sapphire system, Quantel Consultants) as previously described [19]. Photographs were taken directly from the monitor screen, using 400 ASA black and white film.

RESULTS

BrUTP Is Efficiently Incorporated into Nascent RNA by 2-Cell and 4-Cell Mouse Embryos

We first investigated the relevance of BrUTP as a substrate for mouse embryo RNA polymerases at developmental stages where transcription is known to be effective. After 1 h of incubation with the BrUTP precursor, a strong punctuated labeling was observed in nuclei of early 2-cell (Figs. 2A and 2B; 34 hphCG), late 2-cell (not shown), and 4-cell stages (Figs. 2C and 2D; 58 hphCG) outside of the nucleolar area, as judged by DNA staining with Hoechst 33342 specific dye (Figs. 2A and 2C). No signal was observed if BrUTP or the first antibody was omitted or if embryos were digested with RNase A before immunodetection of incorporated BrU (not shown). On the contrary, digestion by RNase-free DNase I did not abolish the BrU-specific labeling (not shown). These results confirmed that the punctuated signal represents newly synthesized RNA. If α-amanitin was added to the culture medium at 10 μg/ml, no signal was observed, suggesting that RNA polymerase II is involved in this synthetic activity. Although only one cytoplasm was microinjected, both nuclei of early (Fig. 2B) and late 2-cell embryos (not shown) were always labeled. In contrast, in all 4-cell embryos analyzed, only two nu-

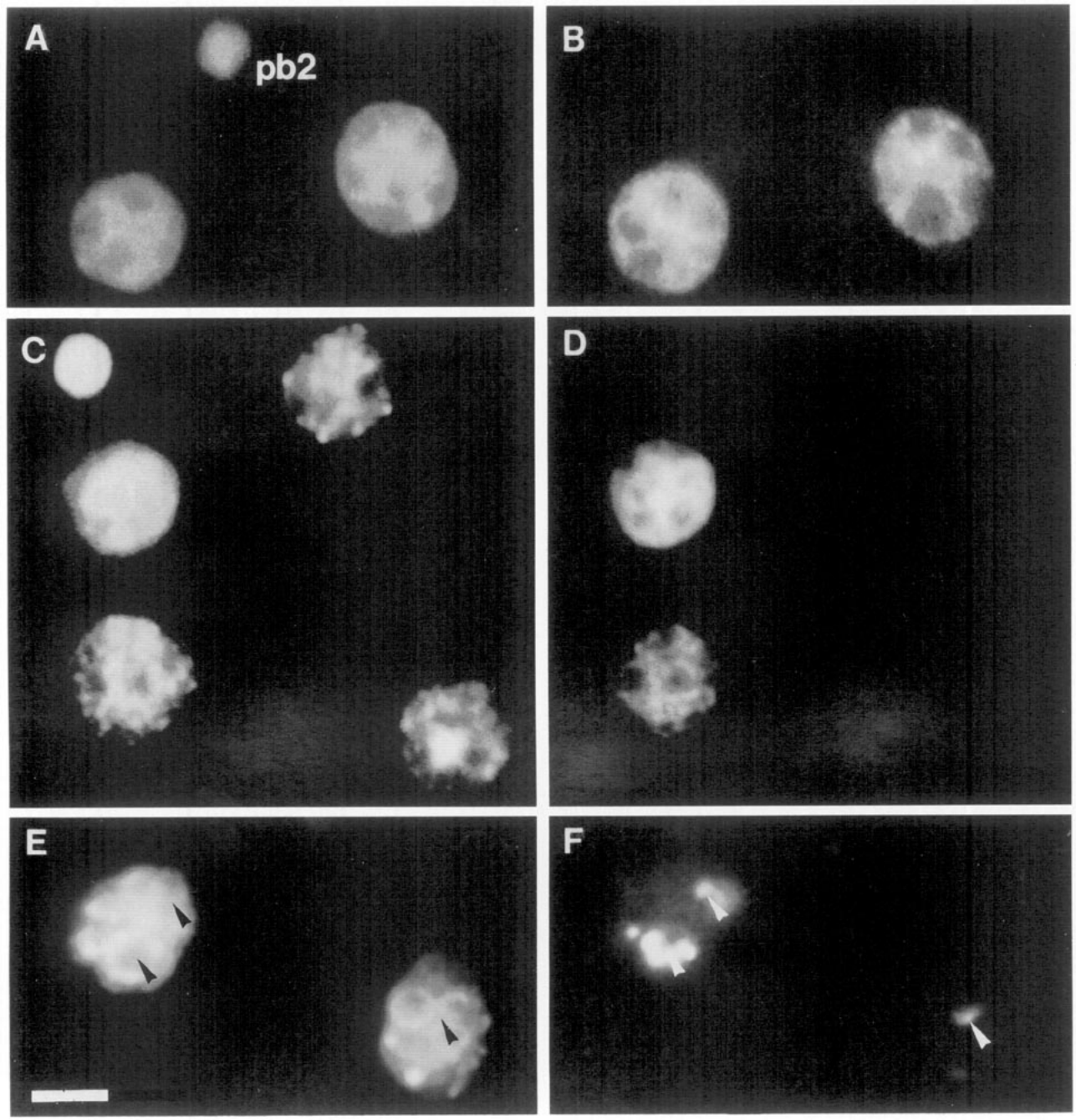

FIG. 2. Localization of BrU-labeled RNA in 2-cell and 4-cell mouse embryos. Live embryos were microinjected with BrUTP, incubated for 1 h, and processed as described under Materials and Methods. DNA staining by Hoechst 33342 (A, C, and E) and immunofluorescent detection of newly synthesized RNA transcripts (B, D, and F) in early 2-cell (A and B) and 4-cell embryos (C and D). Some nucleoli (arrowheads) are labeled at the late 2-cell stage after addition of α-amanitin (E and F). pb2, second polar body. Bar, 10 μm.

clei, supposed to belong to the sister cells, were positive (Fig. 2D). This probably reflects the persistence of cytoplasmic bridges between sister blastomeres of the second mitotic cleavage during the entire 4-cell stage [20].

The lack of BrU labeling in the nucleoli beyond the late 2-cell stage, i.e., at a stage where ribosomal genes transcription becomes active [21, 22], can be explained by an accessibility default of the antibodies to this compartment [13]. However, nucleolar staining can be occasionally observed after α-amanitin treatment of late 2-cell embryos (Figs. 2E and 2F), as already reported in the case of somatic cells [13].

An Endogenous RNA Polymerase II-Dependent Transcription Takes Place in Mouse 1-Cell Embryos

Because of the high sensitivity of this method, we decided to reevaluate the transcriptional activity of 1-cell mouse zygotes. When BrUTP was microinjected at 22 hphCG in early 1-cell embryos, no BrU was detectable in either of the pronuclei even after 1 h of incubation (Figs. 3A and 3B). A negative result was similarly obtained with embryos microinjected at 24 hphCG (not shown). We were, however, able to detect a strong signal when BrUTP was microinjected at 26–29 hphCG in late 1-cell embryos (Figs. 3C and 3D). Among 160 microinjected late 1-cell embryos, 62% showed a punctuated labeling in the entire nucleoplasm of both pronuclei. The pattern of labeling was similar to that obtained on later stages. In some cases, polar bodies were also labeled, as occasionally observed by other authors using different approaches [23–25]. The labeling was completely abolished by α-amanitin co-injection with BrUTP or by RNase A digestion of fixed embryos, but was preserved after RNase-free DNase I treatment (not shown).

These data definitely demonstrate that an endogenous RNA polymerase II-dependent transcription occurs in the late 1-cell mouse embryo, at a time corresponding approximately to the late S or G2 phase (Fig. 1).

Transcription First Starts in the Male Pronucleus

Among the 160 zygotes injected with BrUTP at 26–29 hphCG, 28% did not present any specific BrU labeling, but interestingly, 10% were transcriptionally active in the paternal pronucleus only (as assessed by its size and position with respect to the polar body) (Figs. 2E and 2F). To study more precisely the time dependence of this phenomenon, we injected every hour between 25 and 29 hphCG zygotes collected from a single mouse and reduced the incorporation time to 15 min. We observed that the proportion of zygotes with positive male pronucleus only was maximal (29%, 7 cases among 24) around 27 hphCG. It should be recalled that the post-hCG timing gives only a broad approximation of the development stage of zygotes because of the asynchrony of natural fertilization in the mouse species [16]. This probably explains why we did not obtain more than 29% of embryos with a single positive pronucleus only. However, the relatively high frequency of this event in addition to the fact that we never observed zygotes with only the female pronucleus labeled, strongly suggest that this cannot be the result of an artifact or an abnormal state of development.

A statistical study of the dimensions of pronuclei was performed by image analysis of 1-cell mouse embryos observed *in toto* after Hoechst DNA staining. The main radius of male pronuclei in embryos with two pronuclei transcriptionally positive was 10.9 μm (SD = 0.71 μm, n = 38), while it was only 10.53 μm (SD = 0.66 μm, n = 18) in embryos with only the male pronucleus positive. When analyzed with the Student's test, the difference was significant ($P < 0.05$), but not highly, indicating that the cases where only the male pronucleus is positive are not random, but may correspond to embryos less advanced in development.

Therefore, these results strongly suggest that endogenous transcription takes place first in the paternal pronucleus.

DISCUSSION

By direct *in situ* visualization of transcription sites, we unambiguously demonstrate here that endogenous zygotic transcription by RNA polymerase II begins at the late 1-cell stage in the mouse. It was often previously suggested by indirect methods [8–12], but a direct proof encountered both the low permeability of early embryos to exogenous usual precursors [8] and the probably low level of this activity, as judged by the levels of reporter genes expression [10, 11]. We detect here endogenous transcription in intact mouse embryos from 26 hphCG, i.e., most probably during late S or G2 phases of the first cell cycle. The asynchrony in development after natural fertilization does not allow us to date it more precisely. The transcription sites are present in numerous and well-dispersed small dots all over the nucleoplasm as previously described for somatic cells [13–15], suggesting a similar spatial organization of transcriptionally active domains of chromatin in both cell types. However, one limitation of the present method is that it does not allow a quantitative estimation of the transcription level.

Our work also demonstrates that the male pronucleus is the first to be transcriptionally active. The limited percentage of embryos with positive male pronucleus only (29%) seems to result from the asynchrony in development among embryos and the probable short delay between male and female activation. At least two main hypotheses could account for the difference observed between paternal and maternal pronuclear activities. The first is that the female pronucleus may be transiently less efficient in its ability to import BrUTP from the cytoplasm. The other, which implies a functional or structural difference of the transcription apparatus, is more likely involved. Indeed, a higher ability of the male pronucleus to support transcription of microinjected genes has been reported in cleavage-arrested 1-cell mouse embryos [26, 27] and, more recently, in late 1-cell stage [11]. Other results [28] suggest that the male pronucleus consistently enters S phase before the female pronucleus, but this remains to be confirmed [29]. At the 1-cell stage, the two genomes are physically separated in each

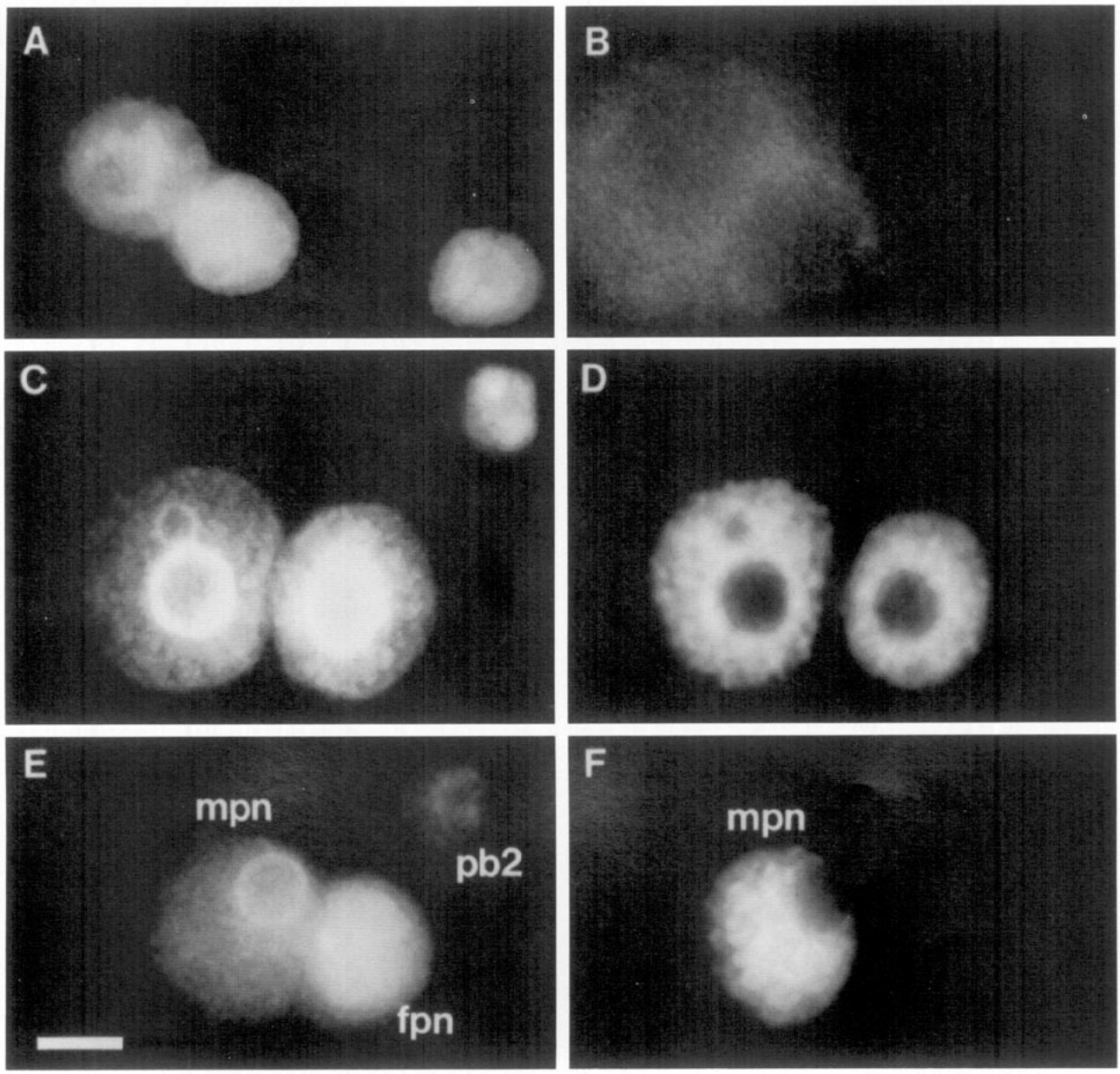

FIG. 3. Localization of BrU-labeled RNA in 1-cell mouse embryos. Zygotes were microinjected with BrUTP at various stages of development, incubated for 1 h (A to D) or 15 min (E and F), and processed as described under Materials and Methods. The corresponding Hoechst staining (A, C, and E) and immunofluorescent detection of transcripts (B, D, and F) are shown. (A and B) 22 hphCG, no transcriptional activity is observed even with excitation intensity and gain of the camera set at maximum. (C and D) 28 hphCG, both pronuclei are labeled. (E and F) 27 hphCG, a case of one embryo with only the male pronucleus (mpn) labeled. The female pronucleus (fpn) is smaller than the male pronucleus and is closer to the second polar body (pb2). Note the increase in the size of the two pronuclei between 22 hphCG (A) and 28 hphCG (E). Bar, 10 μm.

parental pronucleus in the same cytoplasmic environment, which is known to become transcriptionally permissive at the end of the first cell cycle [10]. Therefore, the actual capacity of a given pronucleus to transcribe (or to replicate) the DNA template does not solely depend on cytoplasmic permissiveness but also on the chromatin environment, which appears to be different in male and female pronuclei [27]. This may more likely have its origin in the earliest steps following fertilization, since paternal and maternal chromatins undergo completely different biochemical transformations in their way to formation of nuclei. However, there is prob-

ably no direct link between the transcriptional competence of the two sets of chromatin and the time elapsed after formation of a pronucleus, since the paternal pronucleus is the last to be formed [30].

We probably detect the restoration of a basal activity of transcription at the 1-cell stage, since the need of enhancers observed by reporter gene expression occurs upon 2-cell formation [26, 27]. The nature and fate of the newly synthesized RNA species has now to be determined, since RNA polymerase II synthesizes premessenger RNA, but also most small nuclear RNA. Actually, even after a long incubation time with the precursor (up to 3 h), we could not visualize cytoplasmic export of labeled RNA. However, this does not necessarily indicate that the fate of those molecules is to reside in the nucleus, since it was shown that BrU-substituted RNA is not spliced *in vitro* [31, 32]. In addition, the analysis of nuclear antigens in mouse early embryos suggests that some components linked to the RNA processing machinery are either absent or not properly assembled until the 2-cell stage [33, 34]. If these newly synthesized RNAs have to be processed, the possible lack of a functional nuclear machinery may be responsive for their retention and/or degradation in the nucleus. This may explain why the α-amanitin-sensitive proteins are first detected at the early 2-cell stage [2–4]. Finally, we should point out that these transcripts seem unnecessary to the two first cell cycles, as α-amanitin-treated early 1-cell embryos are able to develop until the late 2-cell stage [3].

In any case, this work will certainly stimulate the study of early development in other mammalian species, where the transition from maternal to zygotic control is assumed to occur at least one or two cycles later [1]. In particular, the method appears to be an extremely useful tool to reinvestigate with a greater sensitivity the nucleocytoplasmic interactions involved in the control of transcriptional activity of individual nuclei in normal embryos and after nuclear transfer experiments.

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REFERENCES

1. Telford, N. A., Watson, A. J., and Schultz, G. A. (1990) *Mol. Rep. Dev.* **26,** 90–100.
2. Flach, G., Johnson, M. H., Braud, P. R., Taylor, R. A. S., and Bolton, V. N. (1982) *EMBO J.* **1,** 681–686.
3. Bensaude, O., Babinet, C., Morange, M., and Jacob, F. (1983) *Nature* **305,** 331–333.
4. Conover, J. C., Temeles, G. L., Zimmermann, J. W., Burke, B., and Schultz, R. M. (1991) *Dev. Biol.* **144,** 392–404.
5. Braude, P., Pelham, H., Flach, G., and Lobatto, R. (1979) *Nature* **282,** 102–105.
6. Bolton, V. N., Oades, P. J., and Johnson, M. H. (1984) *J. Embryol. Exp. Morphol.* **79,** 139–163.
7. Sawicki, J. A., Magnuson, T., and Epstein, C. J. (1982) *Nature* **294,** 450–451.
8. Clegg, K. B., and Piko, L. (1982) *Nature* **295,** 342–345.
9. Clegg, K. B., and Piko, L. (1983) *J. Embryol. Exp. Morphol.* **74,** 169–182.
10. Latham, K. E., Solter, D., and Schultz, R. M. (1992) *Dev. Biol.* **149,** 457–462.
11. Ram, P., and Schultz, R. M. (1993) *Dev. Biol.* **156,** 552–556.
12. Temeles, G. L., Ram, P. T., Rothstein, J. L., and Schultz, R. M. (1994) *Mol. Rep. Dev.* **37,** 121–129.
13. Wansink, D. G., Schul, W., van der Kraan, I., van Steensel, B., van Driel, R., and de Jong, L. (1993) *J. Cell Biol.* **122,** 283–293.
14. Dundr, M., and Raska, I. (1993) *Exp. Cell Res.* **208,** 275–281.
15. Jackson, D. A., Bassim Hassan, A., Errington, R. J., and Cook, P. R. (1993) *EMBO J.* **12,** 1059–1065.
16. Donahue, R. P. (1972) *J. Exp. Zool.* **180,** 305–318.
17. Whitten, W. K. (1971) *Adv. Biosci.* **6,** 129–139.
18. Hogan, B., Costantini, F., and Lacy, E. (1986) Manipulating the Mouse Embryo: A Laboratory Manual, p. 31, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
19. Debey, P., Renard, J.-P., Coppey-Moisan, M., Monnot, I., and Geze, M. (1989) *Exp. Cell Res.* **183,** 413–433.
20. Lo, C. W., and Gilula, N. B. (1979) *Cell* **18,** 399–409.
21. Knowland, J., and Graham, C. (1972) *J. Embryol. Exp. Morphol.* **27,** 167–176.
22. Geuskens, M., and Alexandre, H. (1984) *Cell Differ.* **14,** 125–134.
23. Moore, G. P. M. (1975) *J. Embryol. Exp. Morphol.* **34,** 291–298.
24. Piko, L., and Clegg, K. B. (1982) *Dev. Biol.* **89,** 362–378.
25. Vasseur, M., Condamine, H., and Duprey, P. (1985) *EMBO J.* **4,** 1749–1753.
26. Martinez-Salas, E., Linney, E., Hassell, J., and DePamphilis, M. L. (1989) *Genes Dev.* **3,** 1493–1506.
27. Wiekowski, M., Miranda, M., and DePamphilis, M. L. (1993) *Dev. Biol.* **159,** 366–378.
28. Luthardt, F. W., and Donahue, R. P. (1973) *Exp. Cell Res.* **82,** 143–151.
29. Howlett, S. K., and Bolton, V. N. (1985) *J. Embryo. Exp. Morphol.* **87,** 175–206.
30. Adenot, P. G., Szöllösi, M. S., Geze, M., Renard, J.-P., and Debey, P. (1991) *Mol. Rep. Dev.* **28,** 23–34.
31. Sierakowska, H., Shukla, R. R., Dominski, Z., and Kole, R. (1989) *J. Biol. Chem.* **264,** 19185–19191.
32. Wansink, D. G., Nelissen, R. L. H., and de Jong, L. (1994) *Mol. Biol. Rep.* **19,** 109–113.
33. Dean, W. L., Seufert, A. C., Schultz, G. A., Prather, R. S., Simerly, C., Schatten, G., Pilch, D. R., and Marzluff, W. F. (1989) *Development* **106,** 325–334.
34. Vautier, D., Besombes, D., Chassoux, D., Aubry, F., and Debey, D. (1994) *Mol. Rep. Dev.* **38,** 119–130.

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