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Cell Biology

Cell cycle analysis and X-chromosome inactivation in the developing mouse

(asynchronous DNA replication/embryogenesis)

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ABSTRACT **The duration of different phases of the cell cycle (G_1, S, G_2, and M), as well as the exact time of initiation of asynchronous DNA replication pattern in one of the X-chromosomes in the female mouse during the early stages of development, have been investigated. It was found that early developmental stages in the mouse are devoid of G_1 period, and from the late blastocyst stage the embryonic cells first acquire all the characteristic stages of the cell cycle (G_1, S, G_2, and M). Additionally, asynchronous DNA replication pattern in one of the X-chromosomes in the female embryos was found to be initiated with the appearance of the G_1 period. I have established a correlation between the onset of the G_1 period and the initiation of an asynchronous DNA replication pattern in one of the X-chromosomes of the female mammal during embryogenesis.**

It is well known that cells develop characteristic rates of reproduction during embryogenesis, but the mechanism that underlies such development is virtually unknown. A possible factor controlling macromolecular synthesis may be the length of the time between mitosis and subsequent DNA synthesis (G_1 period). Also, it has been suggested that variability of the cell cycle occurs during this pre-DNA synthetic phase and that G_1 may be absent entirely from the early developmental stages (up to the 16-cell embryo) in the mouse (1). Furthermore, the correlation between the exact time of initiation of the late replication pattern in one of the two X-chromosomes in mammalian embryos and the onset of all phases of cell cycle is lacking.

The present investigations were designed to measure the duration of different phases of the cell cycle in very early stages of embryogenesis and to identify the time of initiation of late DNA synthetic pattern in one of the X-chromosomes in female mouse embryos during early stages of development.

MATERIALS AND METHODS

Superovulation was induced in 6- to 8-week-old ICR/Ha (albino) mice by the injection of 5 international units (IU) of pregnant mare's serum gonadotropin (Nutritional Biochemical Co.) intraperitoneally followed 48 hr later by the injection of 5 IU of human chorionic gonadotropin (Nutritional Biochemical Co.). The mice were subsequently mated. Throughout the entire day after the appearance of the vaginal plug, two-cell embryos were obtained by flushing the fallopian tubes with normal saline; subsequent cleavage stages were found at later times up to the fourth day, when nidation begins. The time schedule of development of the mouse embryos has been reported by Gamow and Prescott (1).

For each cleavage stage, 600 embryos were studied, with a total of approximately 5000 embryos for the entire investigation. The embryos, after collection from the fallopian tubes, were incubated for 20 min at 37° under 5% CO_2 in air in Whitten's medium (2) containing 1 μCi/ml of [^{3}H]thymidine (specific activity 1900 mCi/mmol; Schwarz, Bioresearch, Orangeburg, N.Y.). This procedure was continued every hour for each blastomeric stage (2-cell or 4-cell) until the next one appeared (e.g., 4-cell and 6-cell, respectively).

After incubation of the embryos in isotope medium, they were washed two to three times in nonradioactive medium, followed by normal saline. With use of a small capillary pipette, I placed the embryos on a clean slide. When the saline was just about to dry, a few drops of fixative (3:1 absolute alcohol:glacial acetic acid) were added. The slides were then air dried and extracted with 5% trichloroacetic acid for 15 min at 4° to remove acid-soluble unincorporated isotopic pool. The slides were coated with Kodak NTB_2 nuclear track emulsion; the autoradiographs were developed after 3 weeks of exposure in the dark and stained with buffered Giemsa.

For determining the exact time of initiation of late DNA replication patterns in one of the X-chromosomes in female embryos, the following procedure was followed. Embryos (50–100), all in the same stage of development (2-cell, 4-cell, blastocyst, etc.), were put into Whitten's medium containing 1 μCi/ml of [^{3}H]thymidine (specific activity 1900 mCi/mmol) and incubated for 2 hr. They were then transferred to nonisotope medium containing Colcemid (5 μg/ml) and incubated for another 5 hr at 37°. After this period of incubation, the embryos were washed in normal saline and put into a depression slide containing hypotonic sodium citrate solution (0.7%) for 20 min. They were then put onto a clean glass slide and fixed. The embryos were extracted with trichloroacetic acid (5%, 15 min at 4°) to remove soluble isotopic pools. The slides were coated with NTB_2 nuclear track liquid emulsion and exposed in the dark for 3 weeks, developed, and stained with buffered Giemsa. The slides were scored for labeled metaphase figures, and when such figures were found they were photographed and subsequently degrained to clarify the morphology of the underlying chromosomes for identification. Only the embryos with well-spread metaphases with 40 chromosomes and without Y chromosomes were considered in this study.

In each experiment some slides were incubated in 50 mM Tris·HCl (pH 7.0) plus 10 mM $MgCl_2$ with 100 mg/ml of pancreatic DNase I and 10 μg/ml of snake venom phosphodiesterase to solubilize the [^{3}H]DNA. Slides were incubated at 37° for 2 hr, washed in distilled water, and then extracted with cold 5% trichloroacetic acid. Slides were then re-emulsified and autoradiographs were prepared as described above.

RESULTS

Cell cycle analysis

Although there is variation in the time of estrus and mating, we have observed that in the ICR/Ha strain of mice, each cleavage stage of the preimplantation embryos could be obtained rou-

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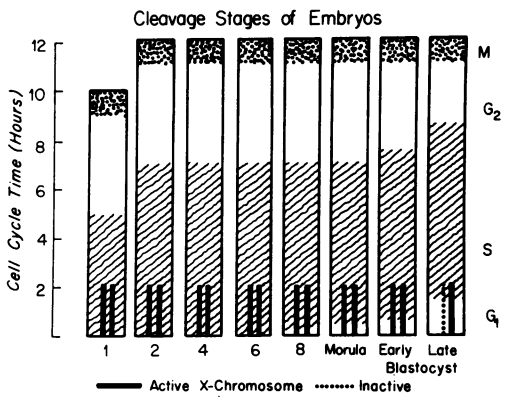

FIG. 1. Length of the cell cycle and appearance of G_1 period and late replicating X-chromosome in the developing mouse embryos. Note the difference in the duration of cell cycle in the single-cell embryo compared to the subsequent stages of development. Also note the synchronous appearance of G_1 period and one of the late replicating X-chromosomes at the late blastocyst stage.

tinely according to the schedule reported by Gamow and Prescott (1). Pronuclear label was observed in single-cell embryos as early as 19 hr after injection of human chorionic gonadotropin, which is approximately 4–5 hr after the formation of the pronucleus. However, the maximum number of embryos with pronuclei in active DNA synthesis occurred at 23 hr and was complete in all zygotes by 31–32 hr. The "S" period was approximately 5 hr, the G_2 period was 4 hr, and mitosis took 1 hr. There was no measurable G_1 that could be observed. At this stage of embryonic development, DNA synthesis was completed in the male pronucleus slightly before the female pronucleus.

In the single-cell embryo the total length of the cell cycle has been estimated to be 10 hr (Fig. 1). In the two-cell stage the "S" period has been estimated to be 7 hr, the G_2 period 4 hr, and mitosis 1 hr. This makes the duration of the cell cycle 12 hr (Fig. 1).

From the two-cell to the late blastocyst stage, the total cycle time remained constant (12 hr). However, there were some variations in the duration of each phase of the cycle (i.e., G_1, S, G_2, and M). There was no evidence of any G_1 period up to the morula stage. After this stage, however, there appeared to be a small pre-DNA synthetic gap (G_1) of approximately 15–20 min. Later on in the late blastocyst stage, particularly when the zona pellucida broke and the embryo migrated to the uterus for nidation, a measurable G_1 period of 1½ hr duration appeared. The G_2 period remained constant from the two-cell to the morula stage and shortened abruptly at the blastocyst stage to make room for the G_1 period. Finally, in the late blastocyst stage, all four phases of the cell cycle appeared (Fig. 1). At this time the total length of the cell cycle measured 12 hr: 1½ hr of G_1, 7 hr of "S", 2½ hr of G_2, and 1 hr of mitosis. This observation was also true for fetuses at the 10th day of gestation.

X-chromosome inactivation

Throughout the cleavage period of the embryos (two-cell to blastocyst), attempts were made to ascertain the exact time of inactivation of one of the X-chromosomes in the female embryos (i.e., embryos without a Y-chromosome in their karyotype) without success. However, in the late blastocyst, especially when the embryo had entered the uterus for implantation and when a clear-cut G_1 period could be measured, one of the X-chromosomes was found to be labeled asynchronously (Figs. 1 and 2). This seems to be the stage of embryogenesis when definite inactivation of one of the X-chromosomes could be ascertained by autoradiography. This pattern was found up to the 10th day of gestation, with a substantial number of X-chromosomes asynchronously labeled.

A summary of the results is presented in Table 1.

DISCUSSION

These results indicate that during the early developmental process, the cells of the cleaving zygotes develop characteristic modes of reproduction. Gamow and Prescott (1) have suggested that up to the 16-cell embryo in the mouse, there is no measurable G_1 period. Our data, in addition to confirming the observations of Gamow and Prescott (1), clearly indicate a total lack of a measurable G_1 period up to the early blastocyst stage. However, after this stage of development, when the embryo moves to the uterus for implantation, a measurable G_1 period can be observed. This is the time, then, when the embryonic cells first acquire all the characteristic stages of a cell cycle (e.g., G_1, S, G_2, and M). The duration of the cell cycle is substantially increased after the first cleavage, and this is principally due to

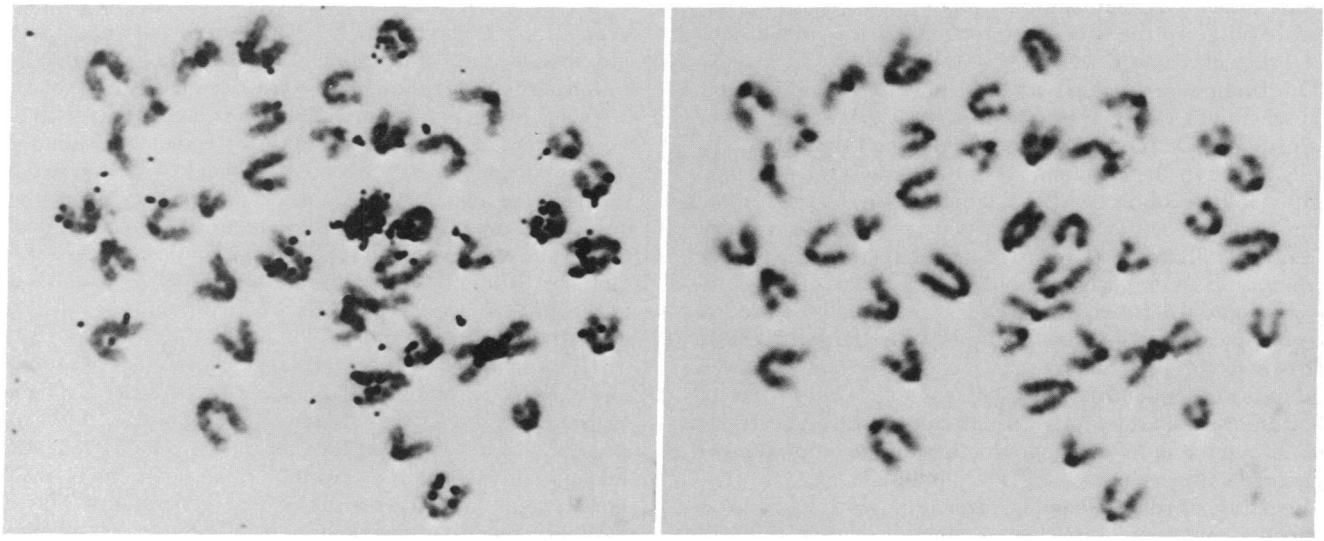

FIG. 2. Autoradiograph of [^{3}H]thymidine-labeled metaphase chromosomes derived from a late female blastocyst. Note the heavily labeled X-chromosome in the middle of the picture on the left. The picture on the right is taken after the autoradiograph was degrained in order to morphologically identify the individual chromosomes.

Table 1. Summary of the pattern of DNA replication in the chromosomes of early developmental stages of mouse embryos

Embryonic cleavage stages	Total no. of embryos (♂ + ♀)	Total well-spread metaphases obtained (♀ only)	No. of metaphases studied (♀ only)	No. of metaphases with synchronous replication of the whole complement	No. of metaphases with only one X-chromosome labeled
1-Cell	300	158	150	150	0
2-Cell	280	200	180	180	0
4-Cell	340	385	350	350	0
6-Cell	320	286	250	250	0
8-Cell	324	315	300	300	0
Morula	310	290	250	250	0
Early blastocyst	326	452	400	380*	0
Late blastocyst	304	482	420	340	80
Implanted blastocyst	364	450	410	164	246
Early fetus (10–12th day of gestation)	155	1345	860	104	756

Well-spread metaphases with 40 chromosomes and without a Y-chromosome were considered to be derived from female embryos.
* Some autosomes not labeled in the remaining 20 metaphases.

the increase in the duration of the "S" period. This increment in the "S" period is partially derived by shortening the length of the G_2 period.

I have established that the onset of a G_1 period during the late blastocyst stage coincides with initiation of the late DNA replication pattern in one of the X-chromosomes. This is a particularly significant observation.

Data bearing on the time of first appearance of a sex chromatin body in the cleaving mammalian embryo in various species have been reviewed by Austin (3). A sex chromatin body has been observed from the blastocyst to the early primitive streak stages with cell numbers ranging from 50 in the pig to thousands in man. Lyon (4) suggested that the appearance of sex chromatin is only an approximate guide to the presence of an inactive X-chromosome; it might be that inactivation had occurred earlier, but that the intranuclear conditions of the cleaving embryo were not conducive to condensation of chromatin. In fact, it has been found in the rabbit that late replication of the X-chromosome appears 24 hr earlier than the sex chromatin (5). Our results could be interpreted similarly, inasmuch as genetic inactivation may have taken place long before we observed the actual late replicating X-chromosomes and/or sex chromatin.

Our findings are in line with those of Gamow and Prescott (1) in that there is no G_1 period during early cleaving embryos in the mouse. Dalcq and Pastiels (6) also found that DNA synthesis occurs in the early part of interphase in the two- and eight-cell stages of rat embryo and in the four-cell stage of the mouse embryo. This latter study also showed that G_1 is absent or very short. Because there are enough data now available for various types of embryos, it could be generalized that cell reproduction during early development, especially before implantation, proceeds without a G_1 period in the cell cycle. In addition to mice, rats, and rabbits, it is also true in *Xenopus* (7) and in sea urchin embryos (8). The absence of the G_1 period allows more rapid cell reproduction and probably indicates the lack of regulation of cell reproduction by the embryo's own genetic machinery.

Recently, Gardner (9) and Gardner and Lyon (10) have described a technique of producing chimeras by injecting cells from one embryo into the blastocele of another. This technique has been refined by Gardner and Lyon (10) to the injection of a single cell, and they have used this method to study X-inactivation. These workers injected cells, which, if female, would be heterozygous for Cattanach's translocation and so would give variegation for albino and wild-type pigmentation, into a blastocyst destined to give mice of uniform distinguishable color, in this case, pink eye. If the differentiation of the two X-chromosomes had already occurred, then the whole cell lineage derived from the injected cell would have the same X-chromosome active and the chimera would have only two colors: pink eye (host) and either wild type or white. If, however, the inactivation of one of the X-chromosomes had not occurred in the injected cell at the time of transfer to the host blastoceles, then the resulting chimera would have three colors: wild type, albino, and pink eye.

Gardner and Lyon (10) injected cells from 3½-day-old embryos into those of the same age and obtained some three-colored and one two-colored chimera. Additionally, they injected cells from 4½-day embryos to 3½-day blastocysts and obtained a similar result, i.e., some three-colored and some two-colored animals. These results are indicative of the two X-chromosomes becoming differentiated at or soon after the stages studied.

Such evidence as there was from other sources suggested to Lyon (4) that in the mouse, X-inactivation occurred at a stage later than 3½ days of gestation (4). Our data indicate that initiation of the late replication patterns occurs late in the blastocyst stage at the time of implantation. This coincides with the 4½ to 5 days of gestation, which supports the observations of Gardner and Lyon (10). Recently Takagi (11) has studied X-chromosome inactivation in the mouse embryos by using quinacrine mustard fluorescence, acetic saline Giemsa technique, and autoradiography. His results indicate that X-chromosome differentiation occurs around the 40- to 50-cell stage of the embryo, which correlates well with our observation of 4½ to 5 days of gestation.

The synchronous onset of a G_1 period and late replication of one of the X-chromosomes in late blastocyst is not surprising, inasmuch as the G_1 period marks the synthesis of RNA and protein, which may be required for further cell differentiation, and perhaps synthesis of repressors that keep one of the X-chromosomes inactive in subsequent generations. This explanation seems reasonable because of the fact that all differentiated cells possess a measurable G_1 period, and therefore the appearance of this phase of cell cycle can be taken as the time of onset of differentiation in the early mouse embryos.

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