X-CHROMOSOME REPLICATION IN EARLY RABBIT EMBRYOS[1]

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IT is generally believed that the sex chromatin which is found in the cells of mammalian females represents a genetically inactive X chromosome (LYON 1961; RUSSELL 1961) which is heterochromatic and is replicated out of synchrony with the other X (ATKINS, TAFT and DALAL 1962; GRUMBACH, MORISHIMA and TAYLOR 1963; OHNO and CATTANACH 1962).

According to the single-active-X hypothesis, inactivation occurs during early embryonic development in mammalian females. The only direct evidence for the time of inactivation has been the appearance of the chromatin body in the embryo. In the mammals whose embryos have been studied, the sex chromatin is generally not seen until sometime during the blastocyst stage (PARK 1957; MELANDER 1962).

To obtain further evidence regarding the time of inactivation, inactivation was directly studied using a second method, autoradiography. Studies were made on the differences between replication patterns of X chromosomes of early female embryos prior to chromatin body formation and of later embryos having chromatin bodies. The results of these studies are the subject of this paper.

MATERIALS AND METHODS

Feral rabbits from San Juan Island, Washington were used in this study. The rabbit was used because it shows distinct chromatin bodies in interphase nuclei of the female, and because it is relatively easy to obtain early rabbit embryos of known age in large numbers.

Continuous label autoradiography was used to study the end of the chromosome replication period in cells of adult male and female rabbits, in 11 and 12-day rabbit fetuses, and in 16 to 128-cell (2 to 3-day) embryos. Adult tissues used consisted of a fibroblast culture from an adult female rabbit kidney biopsy, and sternal bone marrow from both male and female rabbits. Fetal cells were obtained from seven 11 to 12-day fetuses (three female and four male), which were minced and disaggregated with trypsin. Embryos were obtained from female San Juan Island rabbits that were hormonally superovulated either with Pregnant Mare Serum and Human Chorionic Gonadotropin, or with Follicle Stimulating Hormone and Pituitary Luteinizing Hormone. At about 44 hours after mating, the embryos are generally at the utero-tubular junction. The entire oviduct and the adjoining 1 cm of cornu were removed from each side, and the embryos flushed out into a watch glass by culture medium from a capillary pipette inserted into the fimbrial end of the oviduct.

Cells were cultured and labeled in Medium 199 supplemented with 20% fetal calf serum and

antibiotics. The fibroblast culture used was harvested with 0.05% trypsin (Difco 1:250) in buffered saline. For labeling, tritiated thymidine (Schwarz, sp. act. 6.0 C/mMole) was used at a concentration of 1 μC/ml of medium, and Velban (Vinblastine sulfate, Lilly) was added with the label at concentrations of 0.05 μg/ml for embryos, and 0.02 μg/ml for fibroblasts, bone marrow, and fetus cells. After incubation in the label for 4½ hours, adult and fetal cells were harvested, washed with saline, put into hypotonic 1% citrate at 37°C for 15 minutes, fixed in 3 parts methanol: 1 part glacial acetic acid, dropped onto slides, and air dried. Some of the early embryos were put into label immediately after recovery, while others were cultured *in vitro* to about 64 to 128 cells, or late cleavage stage, and then labeled. After labeling *in toto* for 7 to 9 hours, the albuminous layer and zona pellucida were removed from each embryo with 0.5% pronase and fine glass needles. The longer period of labeling in embryos was found to be necessary to obtain labeled metaphases. After removal of the membranes, the embryos were transferred through a saline rinse, hypotonic 1% citrate, fixative, and onto slides. All pipettes and watch glasses used with the embryos were coated with silicone to prevent the sticking of the embryos to the glass. The embryos were handled under a dissecting microscope at magnifications from 7 to 30×.

Kodak NTB-2 emulsion was applied for autoradiography, and the slides exposed 14 days, developed, stained with McNeal's tetrachrome, and scanned for metaphases. Lightly labeled metaphases were photographed, the slides de-grained (FRØLAND 1965), and the marked cells rephotographed.

Chromatin body preparations were made of male and female fibroblasts, and of some 16-cell (2-day) embryos and 5-day blastocysts. Fibroblasts grown on cover slips were fixed in acid-alcohol on the cover slips, dried, stained with 2% acetic orcein, dehydrated in alcohols, and mounted. Sixteen-cell embryos were de-membranated, fixed in acid alcohol, put into a drop of
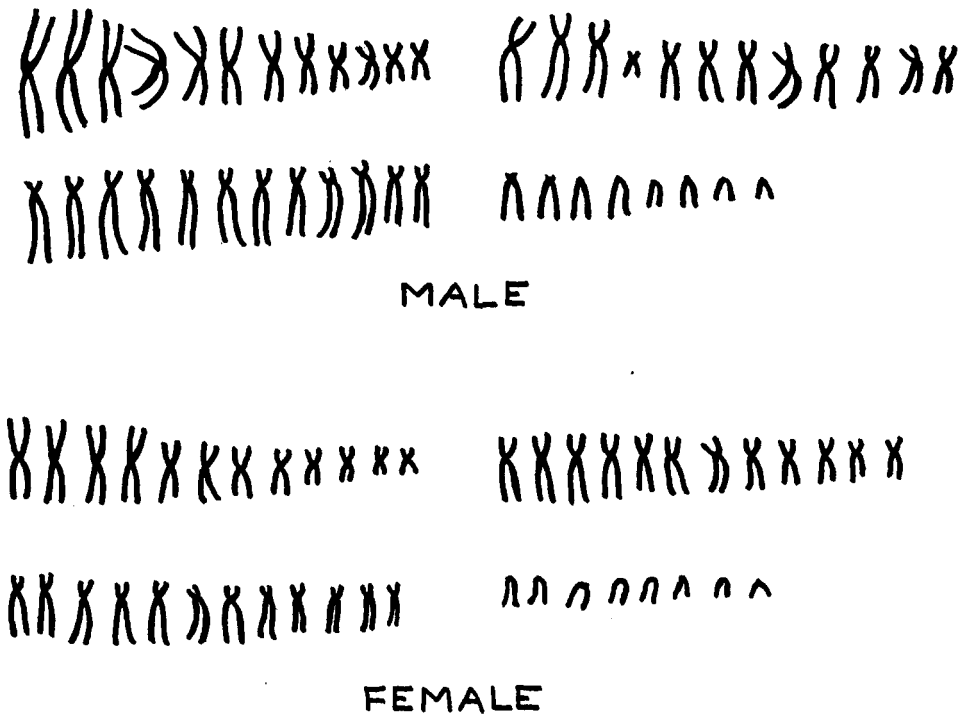

FIGURE 1.—Comparison of male and female karyotypes of the rabbit. Note small, submetacentric Y in male. Tracings of karyotypes.

2% acetic orcein, and a cover slip lightly applied so that its weight would flatten the cells. The blastocysts were fixed, transferred in a drop of fixative to a slide, cut with fine glass needles and flattened out on the slide, where they were allowed to dry. They were then stained with acetic orcein, dehydrated, and mounted.

RESULTS

The rabbit has 44 chromosomes, with six pairs of submetacentrics (Figure 1). The X chromosome of the rabbit, as described by NICHOLS *et al.* (1965), is one of the larger submetacentric chromosomes, with an average arm index of 2.44. The Y chromosome is also submetacentric and is recognizable as the smallest element of the complement, thus allowing identification of the sex of a cell.

By exposing an asynchronously dividing population of cells continuously to a labeled DNA precursor and a metaphase arrestant for a period of time longer than the G-2 (growth) period following DNA synthesis, one can obtain metaphases having different amounts of label on the chromosomes. Cells already in G-2 when label is added will be unlabeled when they reach metaphase, where they are arrested. Cells that are lightly labeled will have incorporated the label into the chromosomes (or chromosomal segments) that were replicating at the end of the synthetic period. This study was based on the chromosome replication pattern at the end of the synthetic period, as indicated by lightly labeled metaphases.

As summarized in Table 1, lightly labeled adult female fibroblasts and bone marrow, and cells of 11 and 12-day female fetuses, usually showed a large submetacentric chromosome that replicated out of synchrony with the rest of this group (Figures 2a, b), so that metaphases having light to moderate amounts of label frequently showed one very heavily labeled chromosome that we interpret to be one of the X chromosomes. In very lightly labeled female cells, often a single chromosome having the morphology of the X was the only chromosome having more than two grains. The adult male bone marrow and the cells of 11 and 12-day male fetuses did not have a corresponding late-labeling large submetacentric chromosome.

Approximately 100, 2 to 3-day embryos (16 to 128 cells) produced about 150 metaphases having light to medium amounts of label, or from 1 or 2 to 20 chromosomes with two or more grains (Table 1). In contrast with the results of labeling in later stages, no lightly labeled cells of these early embryos were found having a single heavily labeled large submetacentric chromosome (Figures 2c, d). Also, no cells were seen having a single chromosome labeled with more than two or three grains.

Although rabbit chromosomes can be divided into four groups according to the position of the centromere, it is difficult to identify individual chromosomes. In lightly and moderately labeled cells, some autosomes were seen which consistently seemed to take up label rather late, although not as markedly so as the late-labeling X in the female. In particular, a pair of large metacentrics and a pair of large sub-acrocentrics, neither in the group including the X, seemed to complete their replication at the end of the synthetic period. This pattern was seen in all stages

TABLE 1

Pattern of X chromosome asynchrony and sex chromatin

Age	Number	No. Light to medium-labeled metaphases	Marked asynchrony of X chromosomes	Chromatin body
2-day (16-cell) embryos	ca. 60	90	—	—
3-day (128 cell) embryos	ca. 30	60	—	—
11 to 12-day ♀ fetuses	3	25	+	+
11 to 12-days ♂ fetuses	4	20	—	—
Adult ♀	3	50	+	+
Adult ♂	1	30	—	—

examined. Unlike the case of the X chromosomes, in which only one of the two X's was late-labeling, both members of a pair of autosomes were labeled to the same extent.

It was found that adult female fibroblasts had distinct chromatin bodies which were not present in male fibroblasts (Figure 2e). Of eight 16-cell (2-day) embryos, none were found to possess sex chromatin. Five of the 11 5-day blastocysts did show distinct sex chromatin (Figure 2f), often associated with a nucleolus. These observations agree with those of MELANDER (1962), who examined preimplantation stages of rabbit embryos from 100 cells (about 3 days) to implantation (6 or 7 days), and found the sex chromatin to originate between 4½ and 5 days of development.

DISCUSSION

According to the single-active-X hypothesis, one of the X chromosomes in each cell of mammalian females is genetically inactivated and becomes heteropycnotic during early embryogenesis (LYON 1961; RUSSELL 1961). This inactivation is thought to be random, irreversible, and permanent in the descendents of any given cell. As summarized by THOMPSON (1965), the main consequences of X-inactivation are dosage compensation for X-linked genes, greater variability of expression of X-linked than of autosomal traits in the female, and mosaicism of females heterozygous for sex-linked genes.

OHNO and CATTANACH (1962), in a study of prophase skin cells from variegated mice having certain coat color genes translocated to the X, presented evidence that the genetically inactive X is also the heteropycnotic one.

TAYLOR (1960) first demonstrated asynchrony of mammalian X chromosome replication in the Chinese hamster. Since then, several mammalian species have been found to have asynchronously replicating X chromosomes in the female (GERMAN 1962; GALTON and HOLT 1965).

The sex chromatin, or Barr body, first described in mammals by BARR and BERTRAM (1949) in the cat, has been shown to represent the heteropycnotic, late-
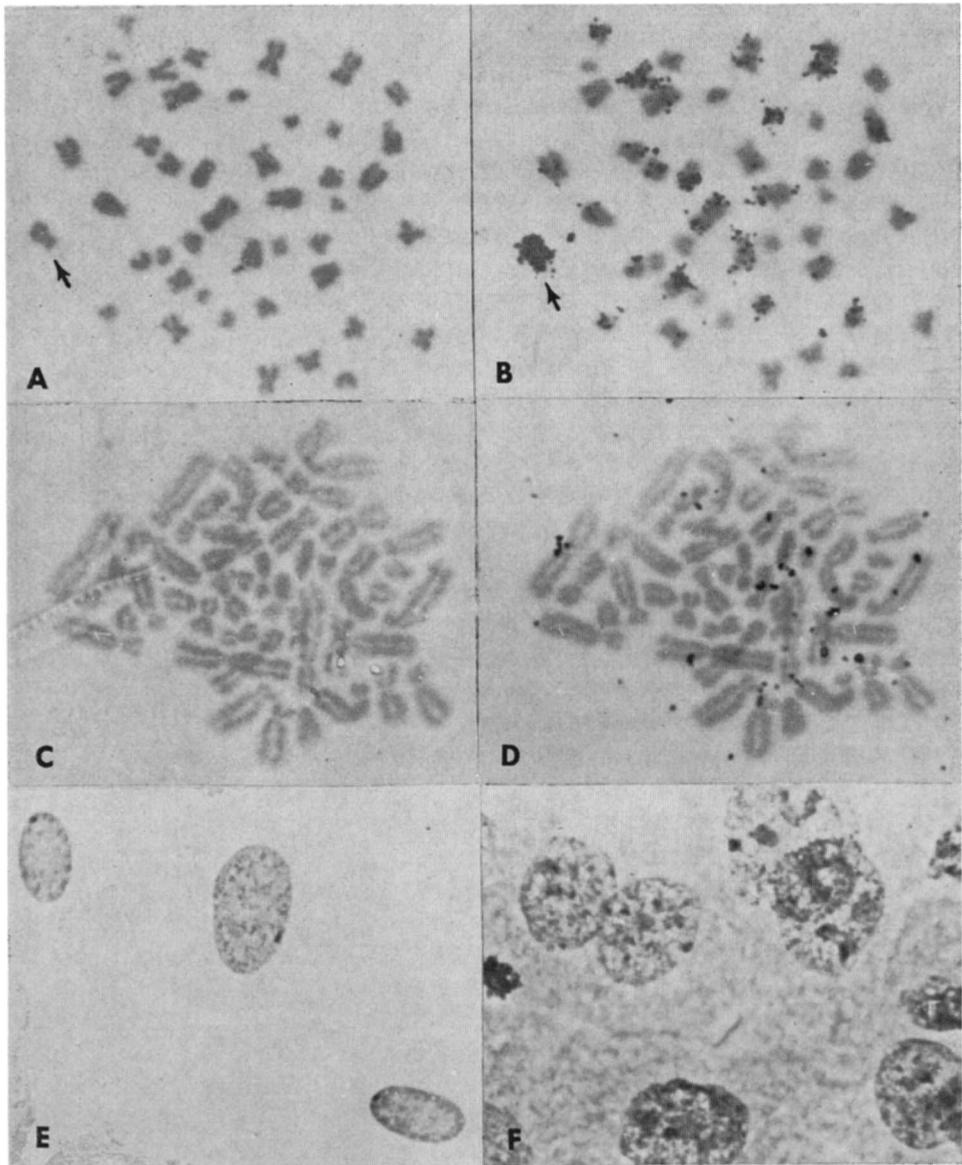
FIGURE 2.—a: Metaphase plate of 11-day female rabbit fetus. 1,240×; b: Autoradiograph of cell shown in a. Note heavily labeled chromosome having morphology of X. 1,240×; c: Metaphase plate of 2-day female rabbit embryo (no Y chromosome present) 1,320×; d: Autoradiograph of cell shown in c. No late-labeling X chromosome present. 1,320×; e: Female rabbit fibroblasts, showing sex chromation. Stained with McNeal's tetrachrome. 720×; f: Five-day rabbit female blastocyst, showing sex chromatin. Stained with acetic orcein. 800×.

replicating X chromosome, which is present in interphase nuclei as a heterochromatic mass. This was demonstrated by labeling studies on cells having excess X chromosomes by ATKINS *et al.* (1962), GRUMBACH *et al.* (1963), and ROWLEY *et al.* (1963).

From diverse sources—genetic, cytological, biochemical—evidence has accumulated which supports the view that the X chromosome that is genetically inactive in each normal female cell is the same X that is late-replicating, heteropycnotic, and forms the sex chromatin.

One important question regarding X-inactivation concerns the developmental time of its occurrence. Until now, the only direct evidence for the time of X-inactivation has been the appearance of the chromatin body in the embryo. In the mammals that have been examined, the sex chromatin is generally first seen in the blastocyst stage, shortly before implantation (GLENISTER 1956; PARK 1957; MELANDER 1962). MELANDER has described the origin of the sex chromatin in the rabbit as occurring in $4\frac{1}{2}$ to 5-day blastocysts. In the present study, sex chromatin was found in 5-day, but not in 2-day, embryos. This is in agreement with MELANDER's work.

Labeling studies on cells of adult rabbits, and on 11 and 12-day rabbit fetuses, showed asynchrony of X-chromosome replication in the female, with one X chromosome replicating late in the synthetic period. There was no corresponding late-labeling X found in the 2 and 3-day embryos examined, or in adult or fetal males. Approximately 150 lightly to moderately labeled metaphases from these early embryos were scanned, and no cells were found having a single heavily-labeled X. Of 16 well spread metaphases karyotyped, nine were female and seven were male. Although it was not possible to obtain an accurate sex ratio of the embryos, since many of them fragmented during treatment, it appears that approximately half the metaphases examined were female.

The observation of markedly asynchronous X-chromosome replication in adult and fetal female rabbits, which show sex chromatin, and the absence of such asynchrony in embryos prior to the appearance of the sex chromatin, indicates that the onset of asynchronous replication may coincide with the onset of heteropycnosis and the appearance of the sex chromatin. In view of the properties of heterochromatin, as recently reviewed by BROWN (1966), this supports the belief that, in female embryos prior to appearance of the sex chromatin, the X has not yet been inactivated, and that this inactivation may indeed occur in the blastocyst, at the time of appearance of the chromatin body. It also appears that, as MELANDER observed, the sex chromatin does appear abruptly at $4\frac{1}{2}$ to 5-days, and that failure to observe it prior to this time is not due to some sort of dispersal effect caused by the large size of the nuclei at this time.

SUMMARY

Cells of adult female rabbits and 11 and 12-day female rabbit fetuses were found to have a late-replicating chromosome having the morphology of an X. This late-replicating X was lacking in cells of male adults and fetuses, and in 16

to 128-cell (2 to 3-day) embryos of both sexes. Since the chromatin body does not appear in the rabbit until 4½ to 5-days, it is believed likely that the onset of asynchronous replication of the sex chromosomes may coincide with the appearance of the chromatin body during embryogenesis.

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