els are considered for the interaction between messenger-RNA molecules required for synthesis of the hybrid enzyme.

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ASYNCHRONOUS DUPLICATION OF HUMAN CHROMOSOMES AND THE ORIGIN OF SEX CHROMATIN

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The sexual dimorphism in diploid intermitotic nuclei first demonstrated in many species of mammals by Barr and his associates[1] has been extensively studied in man and widely utilized in the investigation of anomalies of sex.[2, 3] A heteropycnotic body, the sex chromatin is present in a proportion of human somatic cells of the female and is lacking in those of the male. The sex chromatin is usually planoconvex with its flattened side located against the inner surface of the nuclear membrane. It measures about 1 micron in diameter, stains positively for DNA (deoxyribonucleic acid), and, in some nuclei, has a bipartite structure.

The origin of sex chromatin in man has been a subject of considerable speculation. Barr and his associates[1, 4] originally proposed the useful and widely held theory that sex chromatin represents the somatically associated heterochromatic segments of two X-chromosomes. However, recent evidence obtained by the direct examination of chromosomes is inconsistent with this view and strongly suggests that the sex-chromatin mass in somatic interphase nuclei is derived from a heteropycnotic segment of a single X-chromosome.[5–8]

Based on this evidence that one X-chromosome of the female forms the sex chromatin and from the patterns of mosaicism observed in the expression of sex-linked loci in the mouse, Lyon[9] proposed that the cytological manifestations coincide with a genetic inactivation. To explain the patterns observed, she assumes random inactivation of either the maternally or the paternally derived X-chromosome in an early stage of embryological development. Once the change is induced, the de-

scendants of each cell maintain that state. Grumbach and Morishima,[7] on the basis of sex-chromatin patterns in body cells and cultural isolates of human mosiacs, and Beutler *et al.*,[10] on the basis of enzyme activities in cells of human females heterozygous for sex-linked genes, independently arrived at the same hypothesis. This induction of a heteropycnotic state in the early embryo will be referred to as the "fixed differentiation hypothesis." Genetic evidence[7, 11] does not support the view that the heteropycnotic X-chromosome in the female is derived only from the paternal X-chromosome.[5] In addition, the finding of sex-chromatin-positive nuclei in an XO cell line[6, 7] obtained from an individual with sex chromosome mosaicism suggests that the isopycnotic behavior of one X-chromosome in the female is not attributable solely to suppression of heteropycnosis by a diploid set of chromosomes. The proposed hypothesis does not preclude the possibility of dedifferentiation or differentiation of an X-chromosome in certain cellular environments, for example, in germ cells, embryonic tissues, neoplasms, or long-term tissue cultures.

The evidence supporting the fixed differentiation hypothesis for the origin of sex chromatin in man is based primarily on observation of intrinsic morphologic differences in the two X-chromosomes of the female.[7] It seemed pertinent to seek functional differences in the X-chromosomes which might be related to the differential behavior of the two X-chromosomes in female intermitotic nuclei. Taylor[12] has reported a technique by which it is possible to detect variations in the concentration of radioisotope in structures as small as parts of single chromosomes or even single large molecules of DNA.[13] By the use of tritium-labeled thymidine of high specific activity and autoradiography, differences were demonstrated in the time of replication of DNA within individual chromosomes as well as among different chromosomes in cell cultures of the Chinese hamster.[12] In this species, the X- and Y- chromosomes showed a striking asynchrony in contrast to the other chromosomes. The present report describes the application of this technique to human leucocytes cultured *in vitro*. Evidence is presented which indicates asynchronous duplication of the two "homologous" X-chromosomes in the female and supports the above hypothesis on the origin of sex chromatin in human somatic nuclei. The striking asynchrony of the two X-chromosomes contrasts with the behavior of most homologous pairs of autosomes which appear to be similar in their patterns of replication.

Methods and Subjects.—Cytogenetic studies: Human leucocytes from peripheral blood obtained from six normal subjects and two subjects with sexual anomalies who had abnormal sex chromosome constitutions were cultured *in vitro* by minor modifications of the technique of Moorhead *et al.*[14] Thymidine-H^3 (specific activity 1.88 curies/mM; Schwarz Laboratories, Mount Vernon, New York) was added on the third day in the amount of 1 μc/ml of culture medium. After incubation for 10 min, the cells were washed and reincubated in culture medium containing a large amount of unlabeled thymidine (100 $\times$ the molar concentration of the thymidine-H^3) in order to dilute any unbound thymidine-H^3 remaining in the cells. The cells were fixed 1 to 8 hr after removal of thymidine-H^3, in most instances 3 to 4 hr. Following treatment with colchicine and a hypotonic solution, the preparations were stained by the Feulgen technique and Kodak stripping film AR-10 was applied. After three weeks of exposure, the autoradiograms were developed and the chromosomes were counterstained through the film with azure B bromide.[12]

Cytological studies: Buccal smears were obtained from all subjects and examined for sex chromatin. In the two individuals with abnormal sex chromosome constitutions, vaginal smears and preparations of cultured skin cells were also studied for sex chromatin. Peripheral blood smears were examined for the detection of "drumsticks" in neutrophil leucocytes. The method of Klinger and Ludwig[15] was used for the staining of buccal smears, vaginal smears, and cultured skin cells.

Results.—Many of the cells which reached prophase and metaphase between one and three hours after removal of the thymidine-H^3 had labeled chromosomes in their complement. Labeling of individual chromosomes was not uniform. Presumably depending upon the rate of synthesis of DNA particles at different sites at the time of exposure, regions of a single chromosome varying from small discrete segments to a major portion incorporated sufficient thymidine-H^3 to render them visible by autoradiography. In a number of late prophase and metaphase cells, a majority of the chromosomes were labeled in localized regions. A small proportion of cells in late prophase or metaphase contained only one or a few labeled chromosomes. By comparison with the results reported previously in Chinese hamster cells,[12] we concluded that these cells had almost completed their cycle of DNA synthesis at the time of exposure to thymidine-H^3. Therefore, only those few chromosomes which complete DNA synthesis later than the majority of the complement were labeled when cells were exposed to thymidine-H^3 at a late stage in the synthetic cycle.

In cells of three normal females (sex chromatin-positive) examined thus far, a small proportion of the late prophase and metaphase cells which incorporated thymidine-H^3 2–4 hr before fixation contained one chromosome distinctly more heavily labeled than any other in the complement. In some of these cells, only this chromosome had any detectable label. This late labeling chromosome has the size and morphology of an X-chromosome (Fig. 1). The other X-chromosome was not conspicuously different from the majority of autosomes in its pattern of replication. Less striking asynchrony in DNA replication was found among the autosomes, which, as a group in contrast to the two X-chromosomes, showed similar labeling patterns in homologous pairs of chromosomes (Fig. 2). Portions of either chromosomes 4 or 5 and the major portion of either chromosomes 21 or 22 were labeled later in the interval of DNA synthesis than any except the late replicating X-chromosome. Other labeling patterns that are observed included a terminal sector of one arm of chromosome 1, the major portion of one pair of the group 13, 14, and 15 chromosomes and of one pair of the group 19 and 20, all of which regularly finished replication earlier than the majority of the complement.

The late replicating X-chromosome was not found in cells cultured from three normal male subjects. Their somatic interphase nuclei were sex chromatin-negative.

In a sex chromatin-negative XO female subject[16] with gonadal dysgenesis who has 45 chromosomes in diploid cells, the late labeling X-chromosome was also absent. Additional evidence that the late replicating X-chromosome is the same one which forms sex chromatin comes from the observation that it is found near the periphery of the chromosome complement more often than should occur by chance. This positioning is consistent with the characteristic peripheral location of sex chromatin.

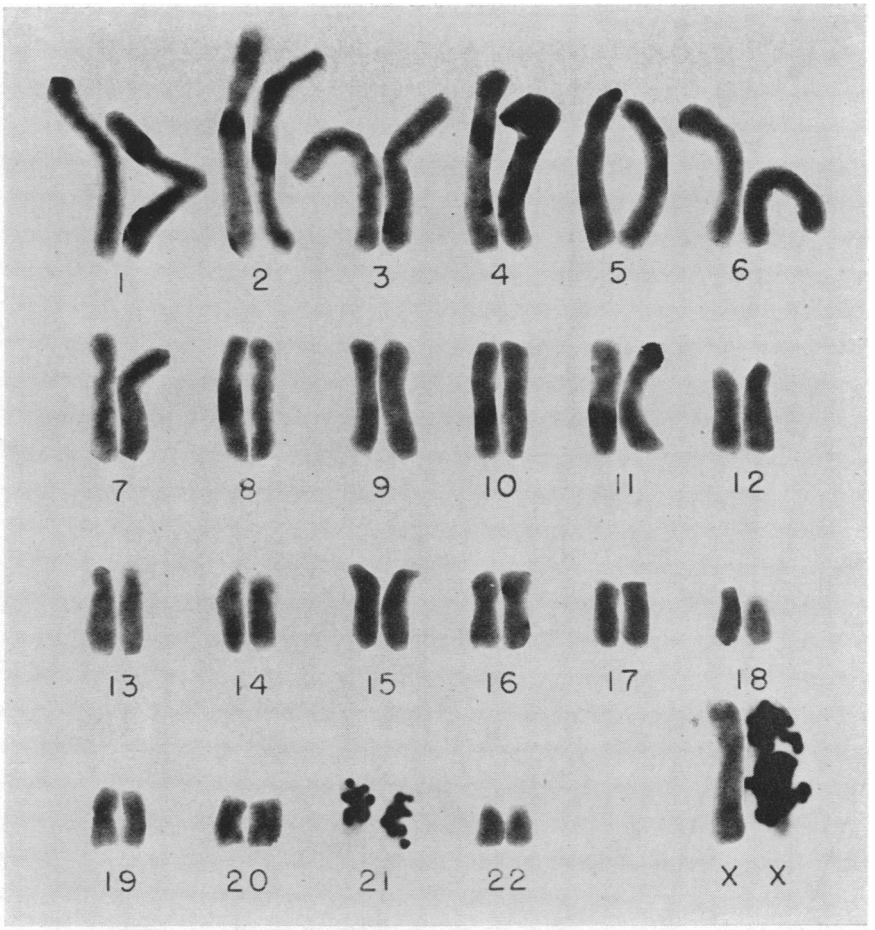

FIG. 1.—Karyotype of a normal female. Prepared from a photomicrograph of the autoradiogram of a metaphase cell exposed to thymidine-H^3. One of the two X-chromosomes is heavily labeled. Note the late labeling pattern of either chromosomes 21 or 22 (tentatively designated as No. 21).

Studies of sex chromatin in a patient with XO/XX/XXX sex chromosome mosaicism have been reported elsewhere.[6, 7] Apparently, the condition arose by nondisjunction in an XX zygote at some mitosis following the first cleavage. It need only be mentioned here that XO cell lines established in tissue culture from two skin biopsy specimens of this patient contained a single sex-chromatin body in a high proportion of intermitotic nuclei, whereas in cell lines with XO/XX/XXX mosaicism derived from a third skin biopsy specimen, single or duplicate sex-chromatin masses were observed in many but not all of the interphase nuclei. Cultures of peripheral leucocytes also showed XO/XX/XXX sex-chromosome mosaicism.[7] In leucocyte cultures exposed to thymidine-H^3, there were a variety of cell types classified on the basis of labeling. Cells with 45 chromosomes, presumably XO, in some instances contained a late labeling X-chromosome, but in other cells a chromosome with this characteristic was not detected. In cells with 46 chromosomes, presumably XX, one late labeling chromosome was usually present. However, in a few cells with 46 chromosomes in which one would expect to find the late labeling

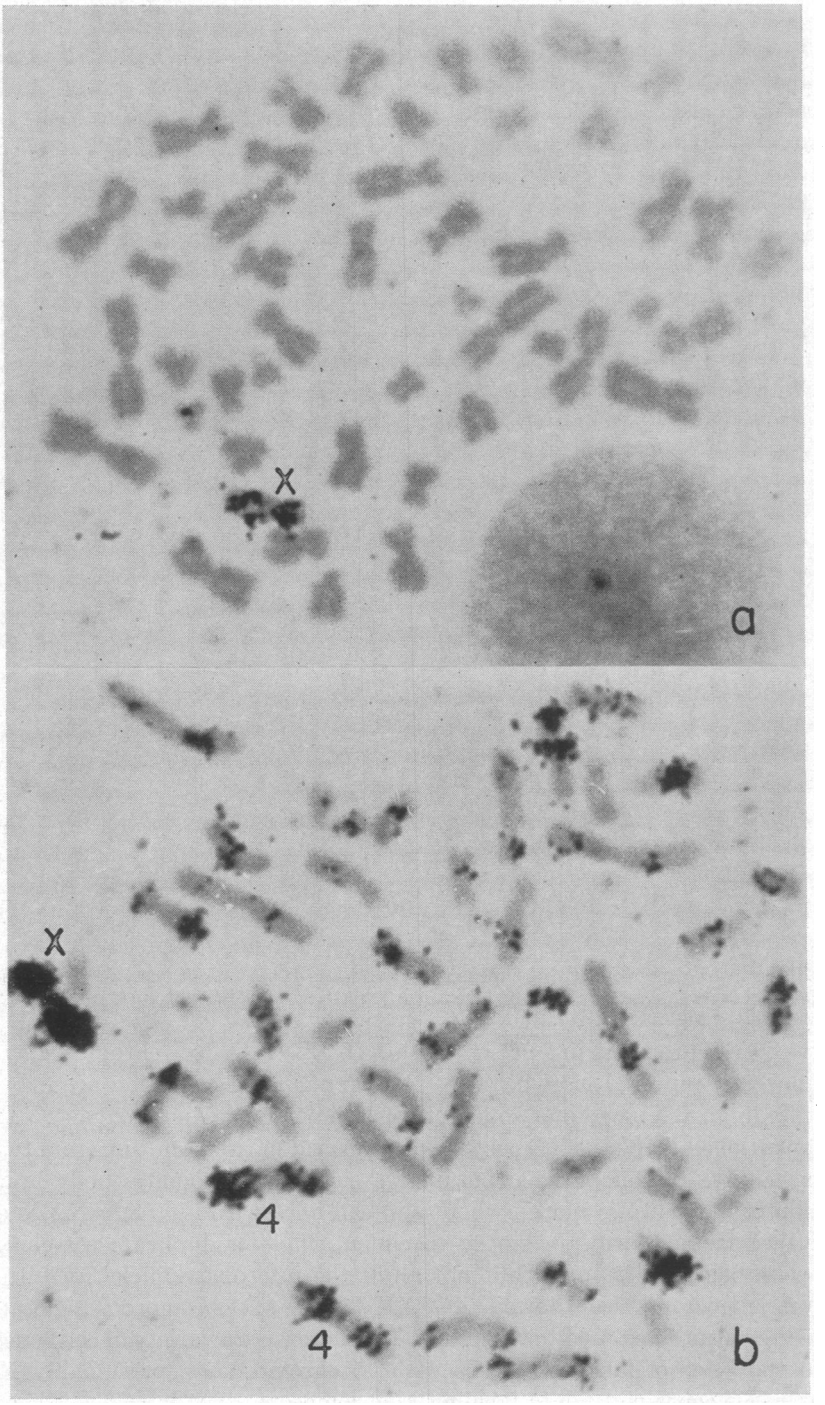

FIG. 2.—Photomicrographs of the autoradiogram of metaphase chromosomes from a patient with XO/XX/XXX sex chromosome mosaicism. (*a*) Only one of the three X-chromosomes was heavily labeled in this cell with 47 chromosomes and an XXX sex chromosome complex. (*b*) One X-chromosome is heavily labeled in a cell with 46. A pair of either chromosomes 4 or 5 (tentatively designated as No. 4) also shows a characteristic labeling pattern.

X-chromosome from the labeling pattern exhibited by the other chromosomes, the characteristic late replicating X was not identified. Although this negative evidence is inconclusive, it is possible that some of the XX cells cultured from this sex chromosome mosaic may lack a late reproducing X-chromosome. In cells containing 47 chromosomes, presumably XXX, there was either one or two late replicating X-chromosomes.

Discussion.—Autoradiographic studies using thymidine-H^3 indicate that the two homologous X-chromosomes in human females have different patterns of DNA replication. One X-chromosome regularly continues its process of replication for a short period in the cell cycle after most of the other chromosomes in the complement have completed their synthesis of DNA. Confirming evidence tentatively identifying a late replicating chromosome in this size group is also being presented by German.[17] The asynchronous replication in the two X-chromosomes in human female somatic cells is similar but different in detail to that described in the Chinese hamster.[12] In the latter species, the whole length of one of the two X-chromosomes and the long arm of the other X-chromosome replicates late in the cycle of DNA synthesis. On the other hand, in normal human females a major portion of only one of the two X-chromosomes appears to replicate late whereas the other X fails to exhibit a conspicuous difference in its time of replication from the majority of the autosomes. In the male hamster, the long arm of the X-chromosome and the whole of the Y-chromosome have a pattern of late replication. However, in human males an appreciable difference was not detected in the time of DNA replication among the X-chromosome, the Y-chromosome, and the majority of the autosomes when studied 3 to 4 hr after exposure to thymidine-H^3.

Less striking asynchronous replication of DNA was observed within and among the autosomes. However, it is noteworthy that the time of DNA replication among readily recognizable pairs of homologous chromosomes was usually remarkably similar as assessed by the thymidine-H^3 technique, in contrast to the two X-chromosomes of the females.

That mammals have two types of X-chromosomes on the basis of their condensation cycle may be adduced from recent evidence.[5, 7] The experimental findings presented in this report suggest that the intrinsic morphologic differences in the X-chromosomes are related to their asynchronous reproduction. The evidence indicates that the late replicating X-chromosome gives rise to the sex-chromatin body in interphase somatic nuclei of the human female and often shows precocious condensation in prophase. It is not present in normal males. The late replicating X-chromosome is regularly present in normal females but was not detected in the chromatin-negative phenotypic female with an XO constitution. In phenotypic males with sexual anomalies such as seminiferous tubule dysgenesis (Klinefelter's syndrome) associated with an XXY, XXYY, XXXY, or XXXXY sex chromosome constitution, sex chromatin is present (reviewed in refs. 7 and 8) and, depending on the number of X-chromosomes, one would expect to find one or more late replicating X-chromosomes. The regular difference observed in the time of replication of the two types of X-chromosomes in the light of evidence previously considered in detail,[7] including the correlation between abnormalities in the structure of the X-chromosome and the morphologic characteristics of sex chromatin, is consistent with the fixed differentiation hypothesis of the origin of sex chromatin from a single X-chromosome.

Genetic evidence[7, 11] and cytogenetic studies make it extremely unlikely that the potentially heteropycnotic state of an X-chromosome in somatic nuclei is either determined by the maternal or paternal origin of the X-chromosome or transmitted as a permament genetic characteristic of a particular X-chromosome from parent to offspring. This implies that the heteropycnotic or isopycnotic condition is an inducible state that can be changed at some stage in the life cycle. Our observations on the XO/XX/XXX individual described here gives additional information concerning the time and characteristics of this induction.

Although somatic cells of XO females regularly have no sex-chromatin body, the XO cells of sex chromosome mosaics such as the XO/XX/XXX individual may have XO cells with a potentially heteropycnotic, late-reproducing X-chromosome. This variation which contrasts with the consistent pattern observed in normal male and female cells and in cells of chromatin-negative XO individuals suggests that the condition is probably induced at an early stage in the embryo. The differentiation of the X-chromosomes conditions the degree and timing of condensation (heteropycnosis), the timing of replication[12] and the expression of genes[9-11] located in or near the heteropycnotic region. Once induced, the state generally persists in the descendants of the specific induced X-chromosome through many generations of somatic cells. However, the condition may be reversible when the chromosomes reproduce in certain cellular environments. A striking example of an alteration in the heteropycnotic behavior of an X-chromosome is illustrated by the observations of Ohno *et al.*[18] during meiosis in human oöcytes. They showed that oöcyte nuclei in contrast to female somatic nuclei are chromatin-negative and the XX-bivalent does not exhibit heteropycnosis. Contrariwise, the XY-bivalent in human spermatocytes is heteropycnotic.[19] Furthermore, sex chromatin has not been recognized in nuclei of human, feline, or simian embryos before the last blastocyst stage.[20, 21] Park[21] has suggested that there is a difference in the time of appearance of sex chromatin in different embryonic tissues. Accordingly, it appears that homologous segments of the same X-chromosome may be isopycnotic or heteropycnotic, euchromatic or heterochromatic and may duplicate early or late in different cellular environments. Although as yet nothing is known of the biochemical transformation which is associated with the change in state, it may represent a fundamental and widespread mechanism for the control of gene action. In the mammals, and perhaps other groups with different dosages of sex-linked genes in males and females, the evolution of such a mechanism may have been necessary to equalize the action of certain genes in the somatic cells of both sexes. Recently, Stern has presented an interesting discussion of the problem of dosage compensation.

Further studies of the pattern of duplication of the human chromosome complement in abnormalities of sex chromosomes and of the autosomes may be expected to contribute to our knowledge of the behavior of the chromosomes during interphase. It has led to an additional criterion for distinguishing the X-chromosome at metaphase and, for example, may serve to clarify the relationship between the extra small acrocentric chromosome associated with mongolism[23] and the Ph[1] chromosome in the same group which has been found in chronic myeloid leukemia.[24]

Summary.—Tritiated thymidine and autoradiography were used to demonstrate two types of X-chromosomes in human leucocytes grown in culture. One of these, interpreted as the X-chromosome and found in normal female subjects, continues

its DNA replication for a longer time than any other chromosome of the complement. Evidence is presented that it is the same chromosome which produces the sex-chromatin body in interphase somatic nuclei of females. The late replicating X-chromosome was not found in normal males who lack sex chromatin or in a chromatin-negative subject with an XO sex chromosome constitution but did occur in various numbers in the cells of an XO/XX/XXX sex chromosome mosaic. The observations seem consistent with the "fixed differentiation hypothesis" of the origin of sex chromatin from a single X-chromosome.

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