EXPERIENTIA

Vol. 23 – Fasc. 11 Pag. 889–984 15. 11. 1967

Heterochromatization in Opossum, *Didelphys virginiana*

By A. K. SINHA

Cytogenetics Laboratory, Texas Children's Hospital, Baylor University College of Medicine, Texas Medical Center, Houston (Texas 77025, USA)

Recent studies on mammalian heterochromatin have aroused much general interest among experimental cell biologists. It has been suggested that one of the *X*-chromosomes of a female forms the sex chromatin mass at interphase and manifests positive heteropycnosis during early prophase[1]. One of the female *X*'s terminates its DNA synthesis out of phase with the rest of the chromosomes of the complement[2–5]. This late-*X* is either maternal or paternal in origin in different cells of the same individual[6]. These findings, in general, are in agreement with the hypothesis[7] of the single-active-*X* chromosome in mammals[8,9].

The mammalian *Y*, which is genetically inert except for some male determining factors[10] and exhibits heteropycnosis by special cytochemical techniques[11], is also late in DNA replication[5,12–14] despite the fact that sex chromatin is not usually seen in males.

A preliminary study on the terminal patterns of DNA replication of the female opossum complement suggested that the late replication of DNA was not limited to the *X*-chromosome of this female mammal[15]. A few of the autosomes were found exhibiting intense synthesis of DNA by the time the *X*-chromosomes had already terminated or were about to terminate replication.

The present paper incorporates the detailed results obtained on both male and female sexes of this animal with regard to condensation of chromosomes during early prophase and the terminal sequences of chromosomal DNA replication. These two aspects of the study were mainly based on chromosome preparations of opossum leucocytes grown in vitro.

Materials and methods

Opossums (*Didelphys virginiana*) were trapped alive in the vicinity of Houston. The animals were kept at the vivarium of the medical school until completion of the studies.

Leucocyte culture. The details of the technique have been presented elsewhere[16]. In brief, buffycoat along with the plasma on top of the opossum peripheral blood was mixed with tissue culture medium (TC 199 Microbiological Associates, Inc., Bethesda, Maryland; 15% fetal calf serum) and incubated at 37 °C in the presence of crude bean extract.

Isotope labeling. The basic technique of adding isotope followed in the present study was essentially the same as has been used for human leucocytes[17]. On the third day of incubation, the growing populations of

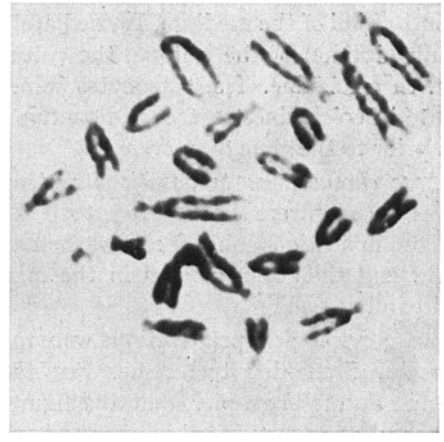


Fig. 1a. A metaphase cell of male opossum (short-term peripheral blood culture).

[1] S. OHNO, W. D. KAPLAN and R. KINOSITA, Expl Cell Res. *18*, 415 (1959).
[2] J. L. GERMAN, Trans. N.Y. Acad. Sci. *24*, 395 (1962).
[3] A. MORISHIMA, M. M. GRUMBACH and J. H. TAYLOR, Proc. natn. Acad. Sci. USA *48*, 756 (1962).
[4] B. B. MUKHERJEE and A. K. SINHA, Can. J. Genet. Cytol. *5*, 490 (1963).
[5] J. H. TAYLOR, J. biophys. biochem. Cytol. *7*, 455 (1960).
[6] B. B. MUKHERJEE and A. K. SINHA, Proc. natn. Acad. Sci. USA *51*, 252 (1964).
[7] H. GRUNEBERG has recently questioned this hypothesis; J. Embryol. exp. Morph. *16*, 569 (1966) and Ann. hum. Genet. *30*, 239 (1967).
[8] M. F. LYON, Nature *190*, 372 (1961).
[9] L. B. RUSSELL, Science *133*, 1795 (1961).
[10] C. STERN, J. med. Educ. *34*, 301 (1959).
[11] M. S. SASAKI and S. MAKINO, Am. J. human Genet. *15*, 24 (1963).
[12] M. GALTON and S. F. HOLT, Expl Cell Res. *37*, 111 (1965).
[13] S. M. GARTLER and B. BURT, Cytogenetics *3*, 135 (1964).
[14] W. SCHMID, Cytogenetics *2*, 175 (1963).
[15] A. K. SINHA, Genetics *54*, 362 (1966).
[16] A. K. SINHA, Experientia *23*, 671 (1967).
[17] S. BADER, O. J. MILLER and B. B. MUKHERJEE, Expl Cell Res. *31*, 100 (1963).

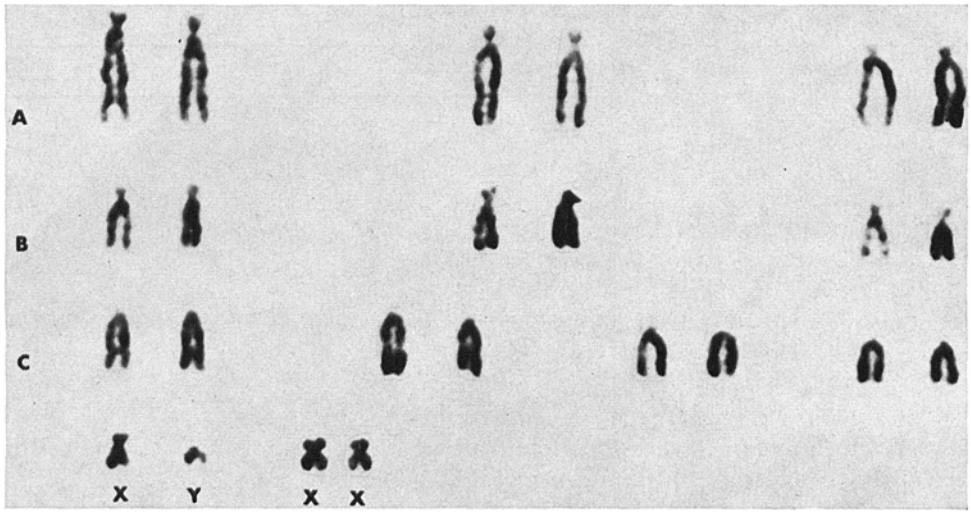


Fig. 1b. Karyotype prepared from the metaphase spread, 1a; *XX* pair being inserted. The individual cells were photographed with Zeiss 100X objective and printed at the same magnifications.

leucocytes were exposed to tritiated thymidine (H^3-TdR, specific activity 3.0 c/m mole, Schwarz Bio-Research, Inc., New York). Each of the cultures received the radioactive DNA precursor in a final concentration 1 μc/ml of the medium. Immediately following the introduction of the isotope, the cultures were treated with colchicine. The leucocytes were allowed to incubate in colchicine-treated, radioactive medium for $4^1/_2$ h prior to the time of harvest.

Metaphase chromosome preparations and autoradiography. The cells were transferred to 1% sodium citrate, fixed in acetic-alcohol (1:3), washed with 45% acetic acid and then resuspended in the mixtures of acetic acid and alcohol.

Temporary cytological preparations were made with acetic orcein, flame-dry technique. For metaphase chromosome autoradiography, suitable figures of unbroken complements were photographed and their locations on the slides recorded. The slides were then dipped in photographic liquid emulsion (Kodak NTB-3), exposed for 5 days, developed and restrained with Giemsa blood stain. Each metaphase figure previously recorded was relocated and only those figures which showed overlying silver grains were rephotographed. Since H^3-TdR was introduced late and remained continuously available to the synthesizing leucocytes until the cultures were terminated, the chromosomes or chromosome segments exhibiting silver grains must represent that part of the total complement which completed DNA replication later than those which did not show any detectable grains.

Prophase chromosome preparation. In addition to utilizing the cultured leucocytes for prophase chromosome analysis, direct preparations were made from bone marrow samples freshly collected from the animals[18]. Colchicine was not added for these cytological preparations in order to avoid any bias resulting from this compound on the condensation of individual chromosomes during prophase stage.

Results

The mitotic chromosomes of opossum. A detailed description of the opossum karyotype has been given elsewhere[19]. The chromosome complement of opossum is composed of 10 pairs of autosomes, a heteromorphic pair of *X* and *Y* chromosomes in the male and an *XX* pair in the female (Figures 1a and b). Although it is difficult to match the individual autosome pair, the *X* and *Y* chromosomes are easily identified respectively as the submetacentric and shortest acrocentric chromosomes of the complement; the *Y* measures approximately half of the total length of the *X*[20].

Differential condensation cycles of prophase chromosomes. In the early prophase nuclei studied, positive heteropycnosis in a number of autosomes and in the

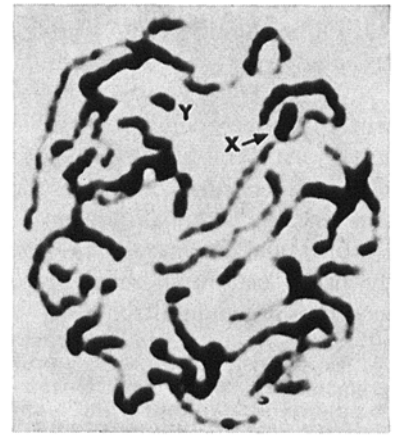


Fig. 2. A male leucocyte showing differential condensation of chromosomes during early prophase. Note heterochromatic nature of *X* and *Y* chromosomes among numerous autosomal heterochromatic elements.

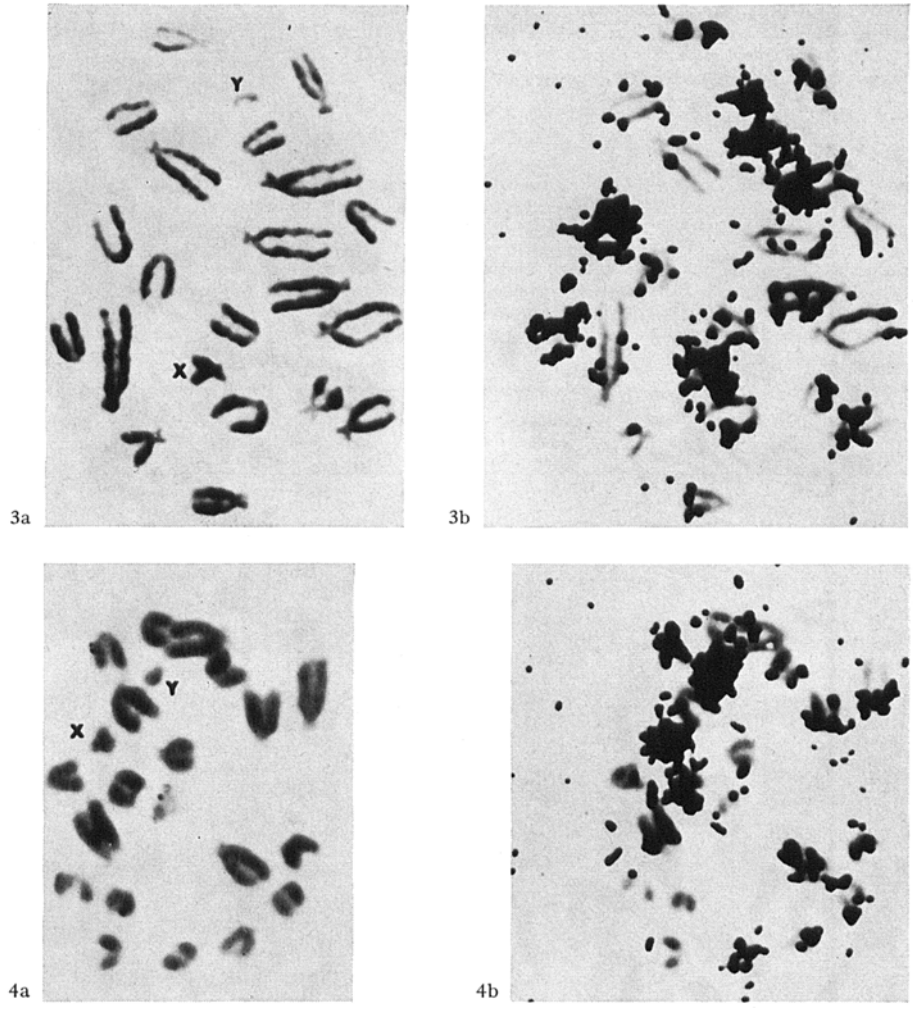
[18] J. H. TJIO and J. WHANG, Stain Technol. *37*, 17 (1962).
[19] A. K. SINHA, Expl Cell Res., in press.
[20] E. L. SHAVER, Can. J. Genet. Cytol. *4*, 62 (1962).

sex chromosomes was clearly observed by their precocious condensation. An entire female X, a major portion of the other female X, a similar portion of the male X, and the whole Y were remarkably allocyclic in behavior. It can be observed in Figure 2 that several autosomes in addition to X and Y chromosomes are displaying heterochromatic areas; some of the autosomes have greater portions of such areas than either X or Y alone. This was found equally true in chromosome preparations obtained either from the cultured leucocytes or the bone marrow cells of the animals.

Terminal sequences of chromosomal DNA replication. From over 100 metaphase autoradiographs examined, all of which were photographed before and after emulsion application, 41% in the male and 39% in the case of the female lent themselves for detailed autoradiographic studies. Intensities of labeling exhibited by these individual metaphases were not the same. They ranged from heavy to light in grain distribution. This indicates that these cells incorporated H^3-TdR at different times during their respective S (synthetic) phases. It was thus possible to reconstruct the terminal sequences of DNA replication of at least those chromosomes that were distinguishable, for instance, the X and the Y.

Patterns of chromosomal DNA replication. (1) *Male complement.* In moderately heavily labeled cells (Figures 3 and 4), the entire X and the Y chromosomes showed heavy accumulation of grains. This was taken as evidence to suggest that at this stage of the S phase, the rate of DNA synthesis of these chromosomes was equally rapid. In addition, a number of autosomes had a high concentration of grains overlying them. Probably, these were the autosomes or autosomal segments which manifest precocious condensation (positive heteropycnosis) during early prophase. Figures 3 and 4, where the labeling patterns of the chromosomes are comparable, further suggest that the distribution of tritium labeled DNA seems strikingly consistent during a particular substage of the S phase.

Further progressive completion of DNA replication can be studied in those cells that showed sequential



3a 3b 4a 4b

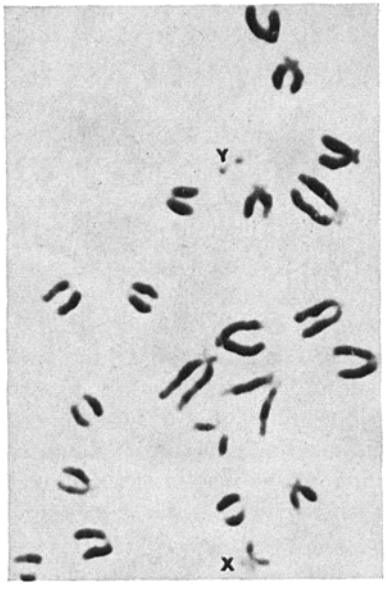
Figs. 3–6. Metaphase leucocytes of male opossum (a) before autoradiography (b) after autoradiography. Interpretations of individual metaphase autoradiographs have been given in the text.

reduction in grain counts. For example, Figure 5 illustrates the DNA synthetic pattern of a cell which came in contact with the isotope during a late stage of replication. At this time, the *Y* was about to terminate replication, whereas the *X* along with a number of autosomes was still engaged in DNA synthetic activities; some of the autosomes had more overlying silver grains than the *X*. In those cells that incorporated H^3-TdR at even later stages of the S phase, as indicated by their light labeling patterns, the *Y* was absolutely free of any detectable grains (Figure 6 and Table I). In Figure 6, it can also be observed that grain distribution in certain autosomes was higher than the *X*-chromosome. During this time, however, the *X*, like some of the autosomes, had not completed replication. Continuation of DNA synthesis in the *X*, after the *Y* had already terminated, obviously suggests a greater amount of heterochromatin in the *X*.

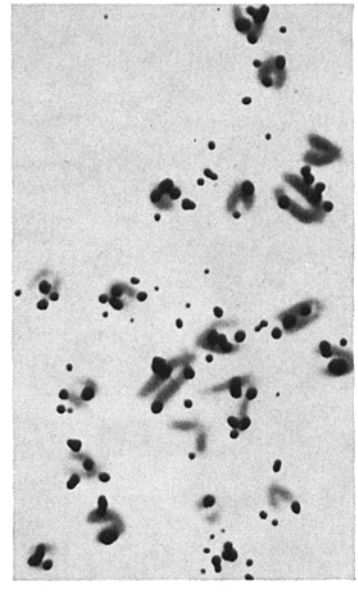
In those figures which showed even lighter grain densities (Table I), a few of the autosomes or autosomal segments were still labeled, whereas the *X*-chromosome was completely unlabeled. Late completion of DNA synthesis in these autosomes, unfortunately not distinguishable from early replicating ones, was consistent with the finding that heterochromatic areas larger than sex chromosomes exist in some of the opossum autosomes as evidenced by early prophase studies.

(2) *Female complement.* The patterns of autosomal DNA replication in the female opossum were not at variance with that of the male. Late synthesizing autosomal blocks were seen equally in the females. Like the male *X* and the *Y* chromosome, the *X*-homologues of the female completed replication earlier than some of the autosomes or portions of certain autosomes (Figure 7 and Table II). Even though opossum female *X*'s seemed to terminate prior to some autosomes, some asynchrony in grain distribution between the *X*-homologues was noted. It should, however, be mentioned here that the grain counts on individual *X*-chromosomes, as shown in Table II, were not strikingly different, presumably because the varying amount of heterochromatin between the 2 *X*'s is not exceedingly great.

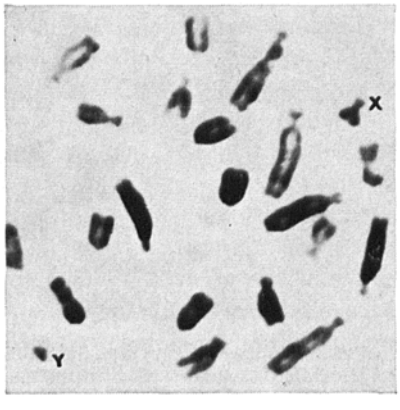
The demonstration of asynchronous but precise patterns of opossum chromosomal DNA replication agrees well with the suggestion of TAYLOR[5] in that the in-



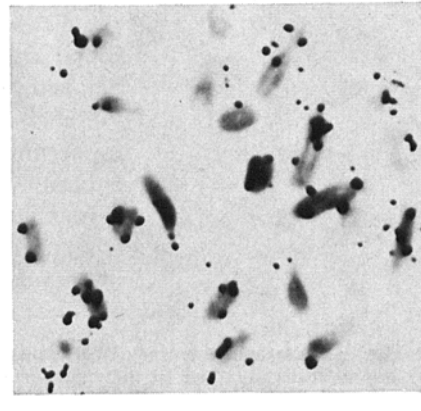
5a



5b



6a



6b

dividual chromosomes of a complement do not replicate at random but are presumably under the control of a highly regulated system which bears an important function in cell duplication. The significance of the disproportionate rate of chromosomal DNA synthesis in other organisms has been extensively reviewed by SCHULTZ[21].

Patterns of interphase nuclei. Male and female labeled interphases were photographed at random. These nuclei exhibited variable degrees of grain densities. With a few exceptions, a majority of the interphases showed several localized areas of intense labeling. Probably these interphase spots (Figure 9) represent the chromosomes which displayed precocious condensation during early prophase and had been replicating late, as evidenced by metaphase-autoradiography.

Discussion

The first indication of asynchrony in *X*-chromosome DNA replication was observed in a grasshopper[22]. In this insect, DNA located in the heterochromatic *X* continued replication later than the DNA in the autosomes. Later, TAYLOR[5] reported a somewhat different asynchronous pattern of replication of the sex chromosomes of the Chinese hamster. The short arm of one *X*-chromosome of the female terminated its replication during the first part of the synthetic period, whereas the long arm of the same *X* and the other entire *X*-chromosome were late in termination. The long arm of the male *X* and the entire *Y* were likewise late to complete DNA replication. In addition, a few of the autosomes showed some delay in their synthesis. Since these reports, autoradiographic studies have confirmed unequivocally the late replicating patterns of the sex chromosomes in a number of mammalian species of which only a few could be cited here[2,12,23–28]. To be more specific, at least in the case of the female, the *XX* female mammals, in general, are seen to possess a single late-labeling *X*-chromosome, i.e., the *X*'s not only show homologue asynchrony but one of the homologues consistently replicates its DNA later than any of the chromosomes of the complement. One should, however,

[21] J. SCHULTZ, Brookhaven Symp. Biol. *18*, 116 (1965).
[22] A. LIMA-DE-FARIA, J. biophys. biochem. Cytol. *6*, 547 (1959).
[23] H. J. EVANS, Expl Cell Res. *38*, 511 (1965).
[24] M. FRACCARO, L. GUSTAVSSON, M. HULTON, J. LINDSTEN, A. MANNINI and L. TIEPOLO, Hereditas *52*, 265 (1964).
[25] M. GALTON and S. F. HOLT, Cytogenetics *3*, 97 (1964).
[26] D. L. HAYMAN and P. G. MARTIN, Cytogenetics *4*, 209 (1965).
[27] N. TAKAGI and S. MAKINO, Chromosoma *18*, 359 (1966).
[28] H. C. TSUENG, V. DEFENDI and P. S. MOORHEAD, Can. J. Genet. Cytol. *7*, 571 (1965).

In order to determine the terminal sequences of DNA replication of the opossum chromosomes, 25 metaphase autoradiographs, where counting of overlying grains was possible, were selected from either sex of the animal. The cells were then arranged in the sequence of increasing grain density.

Table I. The terminal sequences of DNA replication of the male complement. Note that by the time the cells have overlying 50 grains, the *Y* has ceased to replicate but the *X*-chromosome is still active. In cells exhibiting less than 40 grains only the autosomes are seen continuing replication

	Grains per metaphase cell	Grains per *X*	*Y*
1	30	0	0
2	31	0	0
3	40	4	0
4	50	5	0
5	54	7	2
6	59	7	3
7	66	9	2
8	66	9	2
9	72	9	3
10	76	9	3
11	77	9	3
12	77	9	4
13	90	10	4
14	103	10	4
15	106	10	5
16	106	10	4
17	121	11	6
18	129	11	6
19	130	11	6
20	164	12	10
21	166	13	10
22	167	13	10
23	180	15	13
24	191	15	13
25	196	15	13

Table II. The terminal sequences of DNA replication of the female complement. In the cells exhibiting less than 37 grains, the *X*-chromosomes have already completed replication, whereas the autosomes are still engaged in the synthetic activities

	Grains per metaphase cell	Grains per X_1	X_2
1	28	0	0
2	31	0	0
3	33	0	0
4	37	0	3
5	41	0	3
6	45	0	4
7	58	2	4
8	60	1	4
9	65	0–1	5
10	65	1	5
11	66	2	5
12	75	4	6
13	79	4	6
14	87	5	7
15	96	6	9
16	101	7	9
17	103	6	9
18	121	9	10
19	131	9	11
20	133	9	11
21	166	11	13
22	169	11	13
23	181	12	13
24	191	14	15
25	197	14	15

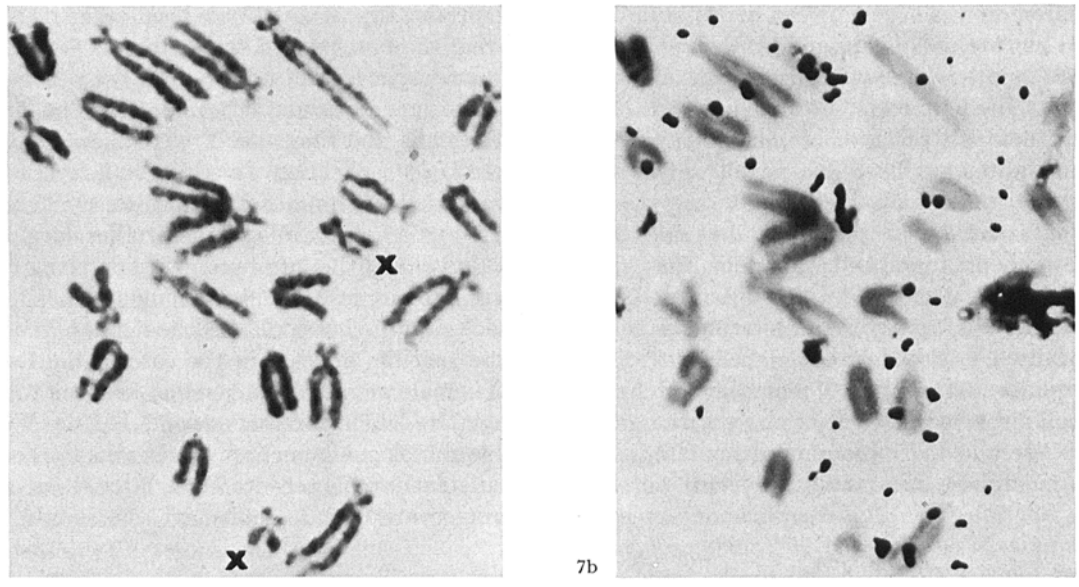
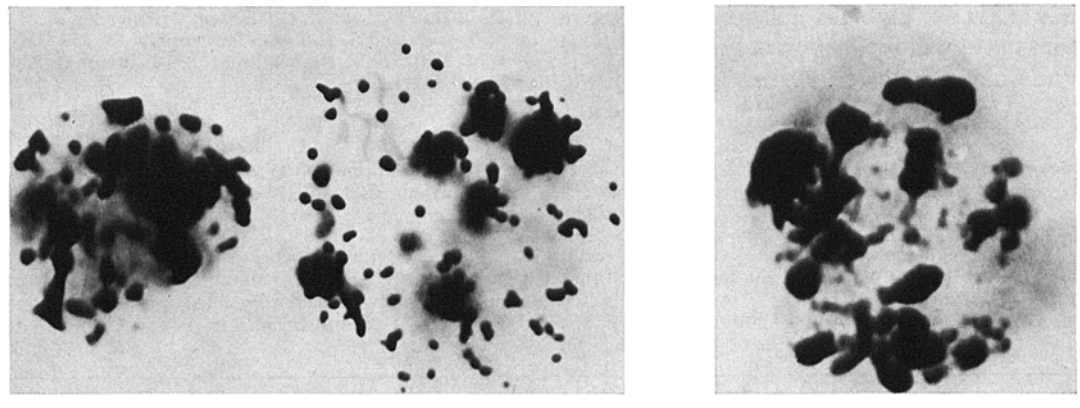


Fig. 7. Isotope was available to this female cell almost at the end of its DNA synthetic period. A few of the autosomes are still showing their DNA synthetic activities whereas the *X*-homologues have terminated replication; (b) is the autoradiograph of cell (a).



Figs. 8–10. Interphase nuclei showing heteropycnotic elements. Note various sizes of heteropycnotic elements.

keep in mind that in none of these mammals were the sex chromosomes found as the smallest pair of the complement. The sequences of termination of DNA replication observed in the opossum chromosomes seem to conform with the general patterns seen in other mammals but differ somewhat in details.

The data presented in this study suggest that like other species, the terminal patterns of DNA synthesis of opossum chromosomes show asynchronous modes of replication. However, there are a few noticeable differences in the manner of opossum chromosome replication that make it unusual compared to other mammalian species thus far studied for autoradiographic patterns of replication. First, in the male the *Y*-chromosome does not appear to complete replication later than the *X*-chromosome. Second, the female *X*-chromosomes, although somewhat asynchronous among themselves, are early replicators. Third, there are certain autosomes in both the complements that continue their synthesis at a time when the sex chromosomes have already terminated or are about to terminate replication. Presumably these are the same autosomes that show comparatively larger condensed (heterochromatic) areas than the sex chromosomes during prophase.

On the basis of the differential behaviors exhibited by the opossum chromosomes, one can derive essentially a common conclusion. In this mammal, the heterochromatization has extended to a larger degree in certain autosomes than the sex chromosomes and it merely becomes a question as to what block of heterochromatin replicates last, a phenomenon that is probably size dependent.

Heterochromatin (secondary constriction) in human autosomes has been identified by special cytological

treatment[29], as well as by its late DNA replication[14]. In addition, in interphase nuclei of leucocytes from the human male and female, LIMA-DE-FARIA and his associates[30,31] have demonstrated the presence of autosomal heterochromatin. Late DNA synthesizing heterochromatic blocks have also been shown to exist in the interphase nuclei[32,33] as well as in certain prophase chromosomes of the mouse[32]. Similarly, in cultured bone marrow cells of the dog, a few of the autosomes are seen to terminate DNA synthesis later than the sex chromosomes[34]. These studies suggest that other mammals as well possess certain amounts of autosomal heterochromatin, but in the case of the opossum this is more pronounced because its sex chromosomes happen to be the smallest pair of the complement.

Considering these species, including the opossum, it is obvious that there is some evolutionary significance in the amount of distribution of heterochromatin between autosomes and the sex chromosomes of mammals. The question as to whether or not the autosomal heterochromatic areas are genetically inactive remains to be answered until additional pieces of genetic evidence become available. Nevertheless, BEUTLER[35] has recently pointed out a few possible autosomal inactive genes in man.

Conclusion

Somatic heterochromatization of the opossum, *D. virginiana*, was studied from prophase and tritiated thymidine-labeled metaphase chromosome preparations. In both sexes of this mammal, a number of prophase autosomes and the sex chromosomes were observed displaying deeply stained condensed areas. These chromosomal areas were interpreted as evidence for heterochromatization. The extent of heterochromatization in the opossum was found much greater in certain autosomes than either the *X* or *Y* chromosome alone. This assertion that some opossum autosomes possess more heterochromatin than the sex chromosomes was supported by data collected on the terminal labeling patterns of the chromosomes. Metaphase autoradiographs prepared from cultured leucocytes of the animals unequivocally suggested that certain opossum autosomes completed replication later than the sex chromosomes. If one assumes that there is heterochromatin in the autosomes, as the evidence suggests, then it merely becomes a question as to what block of heterochromatin replicates last – a phenomenon that is probably size dependent[36].

Résumé. L'héterochromatisation somatique de l'opossum (*Didelphys virginiana*) a été étudiée sur des préparations de chromosomes aux stades de la prophase et de la métaphase, la thymidine ayant été utilisée comme marqueur. Dans les 2 sexes de ce mammifère de nombreux autosomes et les chromosomes sexuels montrent, à la prophase, des plages denses fortement colorées. Ces plages chromosomiales ont été interprétées comme un signe évident d'héterochromatisation. L'héterochromatisation est beaucoup plus accentuée dans certains autosomes que dans les chromosomes *X* ou *Y*. Les autoradiographies de stades métaphasiques dans des préparations effectuées de leucocytes en cultures suggèrent d'une façon non équivoque que certains autosomes de l'opossum achèvent leur réplication plus tardivement que les chromosomes sexuels. Si l'on assume que l'héterochromatine est présente dans les autosomes, comme le démontrent nos observations, il semble évident que l'héterochromatine autosomale a un rôle significatif dans le développement.

[29] E. SAKSELA and P. S. MOORHEAD, Cytogenetics *1*, 225 (1962).
[30] A. LIMA-DE-FARIA, J. REITALU and M. A. O'SULLIVAN, Chromosoma *16*, 152 (1965).
[31] A. LIMA-DE-FARIA and J. REITALU, J. Cell Biol. *16*, 315 (1963).
[32] K. CHURCH, Genetics *52*, 843 (1965).
[33] L. TIEPOLO, M. FRACCARO, M. HULTEN, J. LINDSTEN, A. MANNINI and P. L. MING, Cytogenetics *6*, 51 (1967).
[34] R. C. BROWN, W. L. K. CASTLE, W. H. HUFFINES and J. B. GRAHAM, Cytogenetics *5*, 206 (1966).
[35] E. BEUTLER, Symp. quant. Biol. *29*, 261 (1964).
[36] Supported in part by Baylor University College of Medicine General Research Grant (No. P-13) and grants from the James Picker Foundation and the Texas Heart Association. The author wishes to thank Dr. W. HILL for his help at the vivarium and Miss MARION GAY and Miss SHIRLEY POWELL for technical assistance. The editorial assistance of Miss MARILYN MORNINGSTAR is also appreciated.