Allocycly of the X-Chromosome in Tumors and Normal Tissues*

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SUMMARY

A single, deeply staining heteropyknotic chromosome, most conspicuous during prophase in neoplastic and normal diploid female cells of mouse and rat, is interpreted as one of the two X-chromosomes. Tetraploid female nuclei often contain two such elements, tetraploid male nuclei only one. Tjio and Östergren's (1958) explanation of this phenomenon as a symptom of chromosomal infection with the Bittner milk agent appears untenable. The observed allocycly of the X-chromosome has a bearing on the constitution of the "sex chromatin" in interphase nuclei which is usually composed of the heterochromatin of a single positively heteropyknotic X, rather than two paired X's.

The positive heteropyknosis of a single conspicuously stained chromosome in diploid nuclei of mammary cancers from C3H and DBA mice was recently attributed to viral parasitism (18). Since Tjio and Östergren failed to see a corresponding unit in normal liver and spleen, they postulated an etiologic connection between this chromosomal entity and the origin of milk-agent tumors. By its presence on a chromosome, the Bittner MTA virus supposedly "causes a change of the cell physiology leading to malignancy." The observed monosomic heteropyknosis was interpreted as a symptom of virus infection along an entire chromosome, or of a mutational position effect which is "canalized" longitudinally.

An alternative and more conservative hypothesis—namely, that this *single* heteropyknotic chromosome could be an X—was discarded (18); for every diploid somatic cell of female origin, whether normal or malignant, should contain *two* X's. However, the heterochromatic nature and relative size of the chromosome in question appear to fit the morphologic criteria established for the mouse X (11, 13). We, therefore, considered the possibility that the two homologs of the sex pair may differ in their contraction cycle. This implies an inherent allocyclic tendency of the heterochromatin manifest at any one time through greater compactness and intense staining in only one X in a female diploid nucleus. Allocycly of the X-chromosome was investigated in mitotic figures ranging from early prophase through telophase, in neoplastic and normal tissues, containing or supposedly lacking the milk agent.

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MATERIALS AND METHODS

Chromosomal heteropyknosis was studied in the following tissues: eighteen primary adenocarcinomas of the breast from female mice of the milk-agent strains C3H/StHa, C3H/Jax, DBA1/Jax, and DBA2/Ha; tissue from the 9th serial passage of a transplanted mammary tumor originating in a milk agent-free (8) $C3H_f$/HeHa female; hyperplastic spleen from an Ha/ICR Swiss female mouse infected with Friend virus; clone #2 of the hypotetraploid Ehrlich ascites carcinoma (6); the hypotetraploid EL24 lymphosarcoma ascites, chemically induced in a C57BL male mouse (5); normal lactating mouse mammary gland, mouse Graafian follicle cells, and liver from female mice and rats. The regenerating liver samples were fixed 40 hours after partial hepatectomy.

The preparations for chromosome analysis were made according to two different procedures:

1. The sequence of steps described by Tjio and Östergren (18), which involves 1 hour of colchicine pretreatment *in vivo*, 15–30 min. of soaking in hypotonic 1.1 per cent sodium citrate,

fixation and mincing in acetic orcein, and squashing, as originally recommended by Ford and Hamerton (4).

2. The method of Ohno *et al.* (11) avoids colchicine pretreatment. Small tissue cubes were first soaked for 30 min. in cold distilled water, then fixed in 50 per cent acetic acid for 10 min., minced, and squashed under a silicone-coated coverslip. The coverslips were then separated from the glass slides during a 30-min. immersion in 5–10 per cent aqueous acetic acid solution. Many of the best-spread cells remained affixed to the slide surface and were stained by the Feulgen method, followed by permanent mounting in diaphane.

While the first method was preferable for exact chromosome counting, the second procedure gave a maximum yield of well-spread prophases, wherein heteropyknosis of individual chromosomes could be recognized and photographed.

TABLE 1

CHROMOSOME COUNTS ON NEAR-DIPLOID NEOPLASTIC AND HYPERPLASTIC TISSUES FROM FEMALE MICE

Tissue	Chromosome number (frequency)						Total counts
	<40	40	41	42	43	~80	
Four primary breast tumors from milk-agent strains	4	48	16	7	2	3	80
Breast tumor transplant from milk agent-free strain	2	13	2	1	1	2	21
Friend virus-infected hyperplastic spleen	6	94	17	5	5	2	129

OBSERVATIONS

A heteropyknotic chromosome in primary mammary adenocarcinomas from milk-agent strains.—Eighteen primary breast tumors arising in females of the milk-agent strains C3H/StHa, C3H/Jax, DBA1/Jax, and DBA2/Ha were surveyed for the presence of the single positively heteropyknotic chromosome described by Tjio and Östergren (18). Their observation was confirmed. In most well spread prophase nuclei, this intensely stained chromosome was discernible (Fig. 1). However, in some diploid cells it was represented twice (Fig. 2, 6 o'clock). Heteropyknosis of this entity persisted occasionally into metaphase. In Figure 3, it lies to the left of an extra-long autosome. In a still more advanced metaphase (Fig. 4) distinct heteropyknosis is no longer apparent in any one unit. This also is in agreement with Tjio and Östergren (18), although our C3H and DBA primary tumors showed somewhat greater numerical as well as structural departure from the normal diploid mouse idiogram with its 40 acrocentric chromosomes. Only 60 per cent of the accurate counts in line 1 of Table 1 were exactly diploid. The aneuploid metaphase pictured in Figure 4 has 42 chromosomes, one of which is "new" and matches the extra-long unit of Figure 3 in size.

A single heteropyknotic chromosome in diploid mammary tumor cells from a C3H$_f$/HeHa female.—The C3H$_f$/HeHa strain originated in Dr. Heston's laboratory about 10 years ago. It descended from a litter removed by cesarean section from a high-tumor strain C3H mother and foster-nursed upon a low-tumor strain C57BL female that did not contain the agent. Heston (8) has concluded from extensive tumor data in various sublines that this stock is free of the Bittner milk agent. The incidence of mammary carcinoma in such mice is relatively low, and the tumors arise late in life. Tissue from the ninth transplant generation of a breast tumor originating in one of our C3H$_f$/HeHa females and propagated serially in isologous females had survived 16 months' storage at −78° C. in our frozen tissue bank (7). This "agent-free" neoplasm[1] was re-established in a C3H$_f$/HeHa female for comparison with the C3H/StHa milk-agent tumors.

Aneuploidy and polyploidy in the 10th transplant generation of this breast tumor was no more frequent than in four comparable primary neoplasms (Table 1, lines 1 and 2). Among 22 well spread late prophases and prometaphases examined, 14 demonstrated distinct positive heteropyknosis of a single chromosome (Fig. 5, right of center). Two such units were identified in some tetraploid nuclei, but were less clear in others. In Figure 6, for example, the arrow points toward only one intensely stained U-shaped chromosome, above which lies a somewhat paler straight unit of about the same length.

The size, shape, and staining intensity of the heteropyknotic chromosome were, on the whole, similar in all the mammary carcinoma samplings, whether they originated in milk-agent strains or C3H$_f$/HeHa females.

Persistence of the heteropyknotic chromosome in long-transplanted ascites tumors.—While the chromosome in question was not apparent in several long-transplanted ascites tumors, it was possible to recognize it in clone E2 of the hypotetraploid

[1] Recent electron microscopic studies on such tumors have revealed small numbers of particles resembling the milk agent.

Ehrlich ascites (6) and in the EL24 lymphosarcoma (5). In clone E2, it appears to have persisted for about 50 years since the original transplantation of the parental mammary tumor in Ehrlich's laboratory (Fig. 7).

The occurrence of the positively heteropyknotic chromosome is not limited to tumors derived from females. Although we have not yet seen it in any near-diploid male tumors, it was regularly discernible as a *single* unit in the hypotetraploid EL24 lymphosarcoma ascites which was chemically induced in a C57BL male mouse (Fig. 8). This observation provides a suggestive clue. As a rule, near-diploid female tumor cells and near-tetraploid male neoplasms, both of which supposedly have two X-chromosomes, contain a *single* heteropyknotic unit. Near-tetraploid tumor cells originating in females, and therefore apt to have their normal quota of 4 X's, tend to contain *two* positively heteropyknotic chromosomes (Figs. 6 and 7).

A heteropyknotic chromosome in hyperplastic spleen from a Ha/ICR Swiss female mouse infected with Friend virus.—The acetic orcein fixations for this analysis were made from a greatly enlarged ruptured spleen in a moribund Ha/ICR Swiss female infected with the Friend virus.[2] It is, of course, impossible to distinguish between normal and possibly sarcomatous reticulum cells among diploid spleen mitotic figures many of which had exactly 40 chromosomes (Table 1, last line). Most of the late prophases in this hyperplastic and/or neoplastic tissue contained *one* deeply stained and not yet obviously bipartite chromosome (Figs. 9 and 10). A colchicine telophase (Fig. 11), corresponding to Tjio and Östergren's Figure 6 (18), shows the same unit which here either is doubled over upon itself or represents two closely paired homologs in somatic association.

Identification of the heteropyknotic chromosome as an allocyclic X in normal female somatic cells.—The wide distribution of a similar heteropyknotic chromosome in diverse neoplastic tissues from several mouse strains, either infected with or free of Bittner milk agent, calls for an interpretation other than a localized or indirect viral influence (18) on the substance of a specific chromosome. Another plausible hypothesis has already been proposed in our introduction. The positively heteropyknotic chromosome might be identified as a normal member of the nonmalignant diploid female idiogram. Since it resembles the X both in relative length and heterochromatin content, it justifies the postulate that its unilateral heteropyknosis is the result of X-allocycly. On this assumption, normal diploid somatic nuclei should show, as a rule, only one positively heteropyknotic X, while tetraploid nuclei should contain two, the undiscernible X's being isopyknotic with the autosomes. This is supported by our observations on prophases in Graafian follicle cells, normal mammary tissue, and regenerating liver. The results summarized in Table 2 conform with our tumor data.

Tjio and Östergren's failure to find the heterochromatic unit in normal C3H and DBA mouse liver and spleen (18) might be ascribed to pretreatment with colchicine. While this aids in accurate counting, excessive chromosome contraction would limit the number of good prophases suitable for the study of allocyclic chromosome behavior. In our present work with normal tissues, colchicine pretreatment was, therefore, avoided.

TABLE 2

NUMBER OF ANALYZABLE NONMALIGNANT FEMALE PROPHASES SHOWING 0, 1, OR 2 HETEROPYKNOTIC X-CHROMOSOMES

NORMAL TISSUE FROM C3H/JAX AND DBA/JAX FEMALE MICE	DIPLOID CELLS			TETRAPLOID CELLS		
	0 X	1 X	2 X	0 X	1 X	2 X
Ovarian	31	155	3			
Mammary	2	8				
Liver		25				43

The most acceptable conclusion to be drawn from Figures 12–18 is unilateral positive heteropyknosis (allocycly) of only one X in diploid and two X's in tetraploid nuclei. This finding is not limited to the female mouse karyotype. As previously observed by Ohno, Kaplan, and Kinosita (14), it is equally clear-cut in the rat. A diploid and tetraploid prophase from regenerating female rat liver with one and two conspicuous heteropyknotic units, respectively (Figs. 19 and 20), are here shown for comparison with two similar stages from regenerating mouse liver (Figs. 17 and 18). In diploid prophases of male rat liver, which have only one X chromosome, positive heteropyknosis was not apparent in any of the 42 chromosomes.

DISCUSSION

Intriguing though it is in this epoch of intensified preoccupation with oncogenic agents, the hypothesis that the Bittner milk agent causes

[2] We are indebted to Dr. Charles Campbell of the Department of Pediatrics, Roswell Park Memorial Institute, for supplying the animal infected with the Friend agent.

heteropyknosis in a single chromosome of a female diploid neoplastic nucleus (18) is rendered unlikely by the present findings, because the phenomenon is not restricted to mammary tumors in milk-agent strains. It occurs with equal regularity in breast-tumor tissue from a "milk agent-free" source and persists in certain long-transplanted neoplasms, such as lymphosarcoma EL24. In prophases from some normal female tissues, e.g. Graafian follicle cells and liver parenchyma, it is very pronounced. Furthermore, it is not species-specific for *Mus musculus*, being especially evident in regenerating female rat liver (14).

The single heteropyknotic unit in all these cases is believed to be one of the two X-chromosomes. The relative size of the X is well established from 2nd meiotic metaphase, where it is always conspicuous and ranks fourth or fifth largest (11, 13). A more reliable diagnostic feature than length is its almost entirely heterochromatic nature, there being no autosomes similarly composed.

The occurrence of positive heteropyknosis in only one of the two X-chromosomes of the diploid female nucleus (or in two of the four X's in a tetraploid) is a conspicuous morphologic expression of functional allocycly. The time in the mitotic cycle at which it is most pronounced is prophase. Interphase also shows it periodically.

This phenomenon apparently constitutes the cytological basis for the well-known sexual dimorphism of somatic interphase nuclei in many diverse mammalian species (1, 3, 10). The so-called sex-chromatin has, up to now, been conceived by others and by ourselves (1, 5, 12, 15) as representing the joined heterochromatin of the two X-chromosomes in tight somatic pairing. Indeed, the sex chromatin often seems clearly bipartite; but this is not necessarily true somatic association of two X's. More often, the apparent doubleness results from the tendency of a single heteropyknotic X to fold at the middle (Figs. 11, 15, 17, 18). As phrased by Barr (2), "we are now passing from the descriptive to the more difficult analytical phase in the study of sex chromatin."

The positive heteropyknosis displayed by the heterochromatic portions of chromosomes during certain mitotic stages has often been described as a "heterochromatic" condition. This would imply that positive heteropyknosis and the formation of interphase chromocenters is a *sine qua non* of each heterochromatic region. During male meiosis, both the acrocentric X and Y-chromosomes of the mouse demonstrate positive heteropyknosis of their long arms (13). While it can be said that the X and Y of this species are almost entirely heterochromatic, the positively heteropyknotic condition is usually shown by only one of the two X's in female somatic cells and is not, as a rule, obvious in either the X or Y of male somatic nuclei.

The apparent functional difference between the two essentially isogenic homologs (at least in inbred strains) of the sex-chromosome pair within the same female nucleus poses an intriguing problem. Among the as yet untested explanations for the unilateral heteropyknosis of an X in diploid female somatic cells, two deserve mention:

a) The maternal X is isopyknotic with the autosomes, whereas the paternal X shows positive heteropyknosis.

b) More probably, heteropyknosis alternates between the two X's in a female somatic nucleus regardless of their parental derivation. Depending on stage of DNA synthesis and degree of overlapping in the replication of the two X-chromosomes, one may often discern one X, sometimes no X, or, rarely, two X's (Table 2).

Recent cytogenetic evidence for the existence

FIG. 1.—Diploid prophase from DBA2/Ha primary breast tumor. Arrow points to single heteropyknotic X-chromosome. ×1850.

FIG. 2.—Diploid prophase from DBA2/Ha primary breast tumor. Two heteropyknotic X-chromosomes at 6 o'clock. ×1850.

FIG. 3.—Metaphase from same tumor as in Figures 1 and 2, containing 40 chromosomes. Arrow points to an extra-long autosome, showing structural chromosome change in this "diploid" cell. One X, to the left of the extra-long unit, still exhibits positive heteropyknosis. ×1850.

FIG. 4.—More advanced metaphase from same tumor as in Figures 1–3. No matching homolog for the extra-long autosome (arrow) can be found among the 42 chromosomes of this plate. X-allocycly is no longer apparent. ×1850.

FIG. 5.—Diploid prometaphase with 40 chromosomes from the tenth transplant generation of a mammary adenocarcinoma originating in a "milk agent-free" $C3H_f$/HeHa female. One U-shaped heteropyknotic X lies near the center of this phase-contrast photomicrograph. ×1400.

FIG. 6.—Tetraploid prophase from same tumor as in Figure 5, containing one heteropyknotic X-chromosome (arrow). The straight chromosome directly above this unit may be its homolog. ×1400.

FIG. 7.—Hypotetraploid metaphase (72 chromosomes) from Ehrlich ascites carcinoma clone E2. Two heteropyknotic, somewhat convoluted chromosomes which meet the morphologic criteria for the X are indicated by arrows. ×1400.

FIG. 8.—Hypotetraploid metaphase (78 chromosomes) from the EL24 lymphosarcoma ascites originating in a C57BL male mouse. One strongly heteropyknotic X composed of two chromatids (arrow). ×1400.

of XO female mice (16, 19) and for differential uptake of tritiated thymidine by the X-chromosome in pachytene nuclei of *Menaloplus* (9) hold out promise for an experimental decision between these alternatives.

Is allocycly limited to the almost entirely heterochromatic X-chromosome pair in female mouse and rat nuclei, or is it perhaps also reflected in the behavior of the much smaller heterochromatic regions of the autosomes? During interphase, these are visible as distinct chromocenters, often approximately haploid in number, but ranging all the way to an upper limit of diploid. Thus, the behavior difference between two homologous (and in much of our inbred material, "isogenic") X-chromosomes has significance not only for the cytology of sex chromatin and sex differentiation, but illustrates cyclical heterochromatin function correlated with the reproductive cycle of the cell. As shown by Schultz and his colleagues (17), heterochromatin, even though genetically inert, may nevertheless be "influential in controlling a general nucleic acid synthesis pattern."

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