

# URIDINE-5-H<sup>3</sup> RADIOAUTOGRAPHY OF THE HUMAN SEX CHROMATIN BODY

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## ABSTRACT

To obtain an estimate of the rate of RNA synthesis by the heterochromatic sex chromatin body, human female fibroblasts were labeled with uridine-5-H<sup>3</sup> and radioautographed. The number of grains over the sex chromatin body was compared with the number of grains over a comparable area of euchromatin. The ratio was 0.37. When corrected for the higher content of DNA per unit area in heterochromatin, the *maximum* rate of RNA synthesis by the DNA of the sex chromatin body was approximately 18% of the rate of RNA synthesis by a comparable amount of euchromatin DNA. The rate of RNA synthesis by the sex chromatin body did not increase significantly with partial despiralization of this chromatin at prophase.

The hypothesis that in each female mammalian cell one X chromosome is randomly inactivated (1, 2) explains a great number of phenomena related to the different behavior in males and females of X-linked genes. This inactive X chromosome is detected in the interphase cell as the sex chromatin body (3). It is generally assumed that this heterochromatic X chromosome is inactive in RNA synthesis. This assumption is based, in part, on the observation that heterochromatin in other organisms is largely inactive in the synthesis of RNA (4-6). There are, however, a number of factors which suggest that the inactive X chromosome may synthesize some RNA: (a) some X-linked loci may not be inactivated (7-9); (b) the abnormalities seen in XXY and in XXXXY individuals may be due to incomplete inactivation of the X chromosomes; (c) in mice there appears to be a gradient of X inactivation (10); (d) since heterochromatin may undergo DNA replication without uncoiling (11, 12), it is possible that some RNA synthesis may also occur; (e) electron microscopy of the sex chromatin body reveals it to be considerably less condensed than metaphase chromosomes (13), a feature compatible with some synthesis of RNA;

(f) isolated repressed chromatin is capable of some RNA synthesis (14); and (g) RNA may function as a repressor molecule (15) and thus be a necessary part of the inactivated chromosome.

Thus, although there are valid reasons both for suspecting that in humans the inactive X chromosome is completely inactive, as well as for believing it to be capable of some RNA synthesis, there has been no direct attempt to quantitate its capacity for RNA synthesis. In the present study, to attempt an estimate of this capacity, cultured human female fibroblasts were labeled with tritiated uridine, radioautographed, and the number of grains over the sex chromatin body was compared with the number of grains over a comparable area of euchromatin.

## MATERIAL AND METHODS

Human fibroblasts, obtained from a normal, 46 XX, 11 yr old girl, were cultured on cover slips in Eagle's medium with 10% fetal calf serum, 1-glutamine, sodium pyruvate, nonessential amino acids, penicillin, and streptomycin. The cells were in culture for 4 months and were in their 13th transfer. Following transfer with trypsin, during the log phase of cell growth, uridine-5-H<sup>3</sup>, 50  $\mu$ c/ml (specific activity

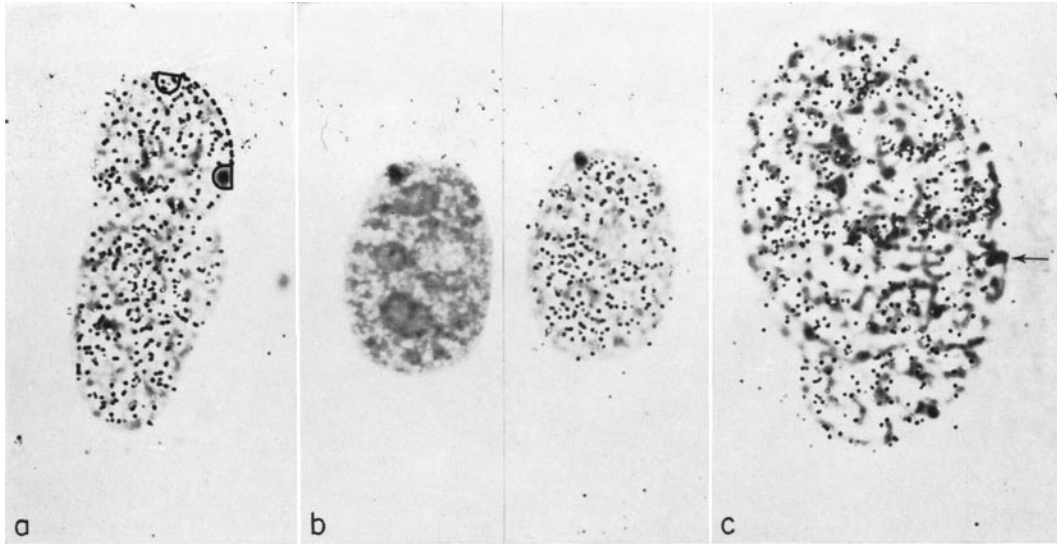


FIGURE 1 *a* The number of grains over the sex chromatin body (in this case, 0) is compared with the number of grains (3) in an identical area shifted a constant distance counterclockwise along the nuclear membrane.

FIGURE 1 *b* Pre- and postradioautography of a uridine-5- $H^3$ -labeled fibroblast.

FIGURE 1 *c* The decrease in the number of grains over the sex chromatin body (arrow) persists into prophase.  $\times 1500$ .

20 c/mm Nuclear-Chicago Corporation, Des Plaines, Illinois) was added to the culture. After 5 min the culture medium was poured off and the cells were fixed for 30 min in several changes of 1:3 glacial acetic acid and methanol. Uridine-5- $H^3$  was used since any conversion to thymidylic acid removes the  $H^3$  label and prevents incorporation into DNA by this route (16). However, because there is some incorporation of label into DNA by conversion to deoxycytidine (17), the cells were treated with deoxyribonuclease, 100  $\mu\text{g}/\text{ml}$  in 5.0 mM  $\text{MgSO}_4$ , phosphate buffer pH 6.7, at 37°C for 2 hr.  $H^3$ -thymidine-labeled control slides demonstrated that DNA was completely removed. The cells were stained in acetoorcein. To avoid selection bias, the cells were photographed before radioautography. 37 cells containing a distinct sex chromatin body located at the nuclear periphery, 14 cells containing neither a sex chromatin body nor other heterochromatin, and 23 cells in prophase were photographed with a Zeiss Photoscope. The cells were covered with Kodak AR 10 fine grain stripping film (18), exposed for 40 days, and developed in Kodak 19b developer. The cells photographed before radioautography were located by coordinates and rephotographed by phase-contrast microscopy. 30 additional postradioautography chromatin-positive cells were photographed. Ribonuclease, 150  $\mu\text{g}/\text{ml}$  (Sigma), pH 6.7 at 37°C for 2 hr, removed all radioactivity.

To evaluate RNA synthesis of the sex chromatin body in relation to nuclear euchromatin, the pre-radioautography photographs were projected, and the sex chromatin was outlined on paper. The radioautographed cell was then projected; the outline of the sex chromatin body was superimposed on the radioautographed chromatin body; and the number of grains within the outline were counted. The outline was then moved a constant interval (5 cm on the projected image) counterclockwise along the nuclear membrane, and the number of grains within the outline was again counted. This gave the number of grains over the sex chromatin body and the number of grains over a comparable area of euchromatin selected without bias (Fig. 1 *a*). Similar counts were made for the cells photographed only after radioautography.

## RESULTS

The results obtained from cells photographed before radioautography and from cells photographed only after radioautography are comparable and are shown in Table I A. The ratio of the number of grains over the sex chromatin body to the number of grains over a comparable area of euchromatin was 0.37. The distribution of grains is shown in Fig. 2.

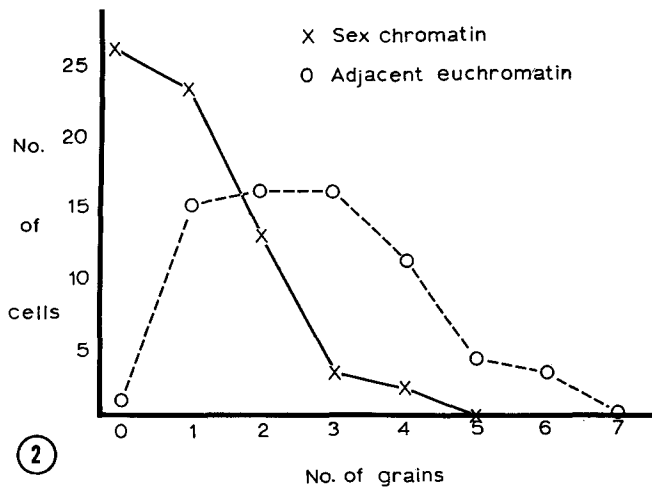


FIGURE 2 The distribution of the numbers of grains over the sex chromatin body and over a comparable area of euchromatin in cells labeled with uridine-5- $H^3$ .

2

TABLE I  
*Uridine-5- $H^3$ -Labeled Human Female Fibroblasts*

A Grains over the sex chromatin body in relation to grains over a comparable adjacent area							
			Grains over sex chromatin (SC)		Grains over comparable adjacent area (AA)		Ratio* SC/AA
			No.	Avg grain count	Avg grain count	SD	
Cells photographed before radioautography	37	0.946	0.94	2.54	1.42	0.372	
Cells photographed after radioautography	30	1.03	1.13	2.76	1.50	0.373	
B Total numbers of grains over nuclei of cells with sex chromatin, of cells without sex chromatin, and of cells in prophase							
			No.	Avg grain count	SD		
Cells with sex chromatin	37		337	160			
Cells without sex chromatin	14		320	104			
Cells in prophase	23		354	137			

\* SC grain count significantly different from AA grain counts  $P < 0.001$ .

Since the suppressor of RNA synthesis in the heterochromatin might be a diffusible factor, the density of grains around the sex chromatin was compared with that around the comparable area of euchromatin. Although some of the sex chromatin bodies showed a decrease in the number of peripheral grains, in others this decrease was not seen and was not sufficiently common to be significant.

There was no significant difference ( $P = 0.65$ )

between the total number of grains over the nuclei of cells containing sex chromatin and the total number of grains over the nuclei of cells without sex chromatin (Table I B).

To evaluate the possibility that, with partial despiralization of the sex chromatin body during prophase, this chromatin might become active in RNA synthesis, 23 cells in prophase were also photographed before radioautography. In those cells which showed an identifiable presumptive

heterochromatic X chromosome, the number of grains overlying it was again less than that over adjacent chromatin (Fig. 1 c). The total number of grains over the nuclei of prophase cells was not significantly different ( $P = 0.55$ ) from that over the nuclei of interphase cells (Table I B), indicating that the suppression of RNA synthesis which occurs during mitosis (19, 20) has not yet begun at prophase.

#### DISCUSSION

Even though the sex chromatin body is located at the thinned-out edge of the nucleus, it is possible that some euchromatin may lie above or below it. Electron micrographs of the sex chromatin body show it to be liberally interspersed with euchromatin material (13). Whether this is euchromatic X chromatin material or autosomal chromatin is not known. Since some non-X euchromatin is probably present in the area of the sex chromatin body, and because tritium has a beta track of up to  $5 \mu$ , the ratio of 0.37 for grains over the sex chromatin body in relation to grains over a comparable adjacent area of euchromatin is a maximal figure. Since the amount of DNA in heterochromatin is approximately twice that in a comparable area of euchromatin (21), the maximum rate of synthesis of RNA by sex chromatin DNA becomes approximately 18% of the rate of RNA synthesis by a comparable amount of euchromatin DNA. Again, since some autosomal euchromatin may be present in this area, the true percentage may range anywhere between 0 and 18%.

#### BIBLIOGRAPHY

1. LYON, M. F., Gene action in the X-chromosome of the mouse (*Mus musculus L.*) *Nature*, 1961, **190**, 372.
2. BEUTLER, E., YEH, M., and FAIRBANKS, V. F., The normal human female as a mosaic of X-chromosome activity: studies using the gene for G-6-PD-deficiency as a marker, *Proc. Nat. Acad. Sc.*, 1962, **48**, 9.
3. BARR, M. L., and BERTRAM, E. G., A morphological distinction between neurones of the male and female, and the behavior of the nucleolar satellite during accelerated nucleoprotein synthesis, *Nature*, 1949, **163**, 676.
4. HSU, T. C., Differential rate in RNA synthesis between euchromatin and heterochromatin, *Exp. Cell Research*, 1962, **27**, 332.
5. BERLOWITZ, L., Correlation of genetic activity, heterochromatization, and RNA metabolism, *Proc. Nat. Acad. Sc.*, 1965, **53**, 68.
6. LITTAU, V. C., ALLFREY, V. G., FRENSTER, J. H., and MIRSKY, A. E., Active and inactive regions of nuclear chromatin as revealed by electron microscope autoradiography, *Proc. Nat. Acad. Sc.*, 1964, **52**, 93.
7. GORMAN, J. G., DIRE, J., TREACY, A. M., and CAHAN, A., The application of  $-Xg^a$  anti-serum to the question of red cell mosaicism in female heterozygotes, *J. Lab. and Clin. Med.*, 1963, **61**, 642.
8. CHILDS, B., Genetic origin of some sex differences among human beings, *Pediatrics*, 1965, **35**, 798.
9. BEUTLER, E., Gene inactivation: the distribution of gene products among populations of cells in heterozygous humans, *Cold Spring Harbor Symp. Quant. Biol.*, 1964, **29**, 261.
10. RUSSELL, L. B., Mammalian X-chromosome action: inactivation limited in spread and in region of origin, *Science*, 1963, **140**, 976.

In relation to the distribution of grain counts over the sex chromatin body (Fig. 2), the lack of a bimodal curve with most counts zero and with a smaller number of counts of 2 or more grain. suggests that whatever activity is present is not due to the synthesis by a few sex chromatin bodies of significant amounts of RNA and the absence of synthesis by others in a different portion of the cell cycle. A minor degree of bimodality could, however, be missed. The existence of some cells with fairly high grain counts over the sex chromatin body suggests that the lower limit of RNA synthesis may be greater than zero.

The absence of a significant difference between the total number of grains over the nuclei of cells without a sex chromatin body and the total number of grains over the nuclei of cells with a sex chromatin body suggests that cells without sex chromatin are not merely dying or degenerate cells.

Due to the conditions of the experiment, utilizing a short pulse of a high concentration of uridine-5- $H^3$ , RNA with a more rapid rate of turnover would be preferentially labeled. Studies are in progress to detect the possible synthesis of a stable RNA as an integral part of the sex chromatin body.

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11. COMINGS, D. E., The persistence of heterochromatin during DNA replication, to be published.
12. HENDERSON, S. A., RNA synthesis during male meiosis and spermiogenesis, *Chromosoma*, 1964, **15**, 345.
13. WOLSTENHOLME, D. R., Electron microscopic identification of sex chromatin bodies of tissue culture cells, *Chromosoma*, 1965, **16**, 453.
14. FRENSTER, J. H., Nuclear polyanions as de-repressors of ribonucleic acid, *Nature*, 1965, **206**, 680.
15. BONNER, J., *The Molecular Biology of Development*, New York, Oxford University Press, Inc., 1965.
16. HAYHOE, E. G. J., and QUAGLINO, D., Autoradiographic investigations of RNA and DNA metabolism of human leucocytes cultured with phytohaemagglutinin; uridine-5- $H^3$  as a specific precursor of RNA, *Nature*, 1965, **205**, 151.
17. COMINGS, D. E., Incorporation of tritium of uridine-5- $H^3$  into DNA, *Exp. Cell Research*, in press.
18. SCHMID, W., DNA replication patterns of human chromosomes, *Cytogenetics*, 1963, **2**, 175.
19. TAYLOR, J. H., Nucleic acid synthesis in relation to the cell division cycle, *Ann. New York Acad. Sc.*, 1960, **90**, 409.
20. FEINENDEGEN, L. E., and BOND, V. P., Observations on nuclear RNA during mitosis in human cancer cells in culture (HeLa- $S_3$ ) studies with tritiated cytidine, *Exp. Cell Research*, 1963, **30**, 393.
21. BERLOWITZ, L., Analysis of histone in situ in developmentally inactivated chromatin, *Proc. Nat. Acad. Sc.*, 1965, **54**, 476.