The hyperactive X chromosome is not early replicating in mitotically active somatic cells of *Drosophila nasuta* males

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Abstract: The temporal order of replication of the X chromosome(s) in mitotically dividing male and female cells in early embryos and in brain ganglia of *Drosophila nasuta* larvae was examined using [^{3}H]thymidine pulse labelling and autoradiography. Both the X chromosomes in female cells and the single X chromosome in male cells replicated in complete synchrony with the autosome set in the nucleus. Thus, unlike the well-known early completion of replication by the hemizygous X chromosome in polytene nuclei in the salivary glands of male *Drosophila* larvae, the single X chromosome in mitotically dividing cells does not replicate earlier than the autosomes. We conclude that transcriptional hyperactivity of the single X chromosome required for dosage compensation in somatic cells of male *Drosophila* is not dependent upon its early replication.

Key words: dosage compensation, hyperactive X chromosome, early replication.

Résumé : Le moment auquel s'effectue la réplication de chromosomes X lors de la mitose dans les cellules de jeunes embryons et de ganglions cervicaux chez les larves du *Drosophila nasuta* a été examiné par marquage court ("pulse") à la [^{3}H]thymidine suivi d'autoradiographie. Les deux chromosomes X chez les femelles ainsi que l'unique chromosome X chez les mâles étaient répliqués en même temps que le jeu d'autosomes dans le noyau. Ainsi, au contraire du chromosome X hémizygote dont la réplication est précoce dans les noyaux polytènes des glandes salivaires de larves mâles du *Drosophila*, l'unique chromosome X présent dans les cellules en mitose n'est pas répliqué avant les autosomes. Les auteurs en concluent que l'hyperactivité transcriptionnelle du chromosome X, requise pour compenser sa présence en un seul exemplaire dans les cellules somatiques du *Drosophila* mâle, ne dépend pas d'une réplication précoce.

Mots clés : compensation de dose génique, chromosome X hyperactif, réplication précoce.
[Traduit par la rédaction]

Introduction

Dosage compensation for X-linked genes in males and females of *Drosophila* spp. is known to operate by hyperactivation of the single X chromosome in somatic cells of males (Mukherjee and Beermann 1965). The hyperactivity of the hemizygous X chromosome is directly manifested in polytene nuclei of the salivary glands of male larvae by the enlarged width, paler staining, and greater [^{3}H]uridine uptake of the hemizygous X chromosome when compared with autosomes or the paired X chromosomes in the female (Mukherjee and Beermann 1965; Lakhotia and Mukherjee 1969) and by the specific association of certain proteins (Kuroda et al. 1991; Palmer et al. 1993). A greater transcription of X-linked genes in other somatic cells of male *Drosophila* has been directly documented through many studies dealing with specific gene transcripts or protein products (Lucchesi and Manning 1987).

It is also well established that the hemizygous X chromosome in polytene nuclei of the salivary glands of male larvae completes a given endoreplication cycle faster and earlier than autosomes in the nucleus (Lakhotia and Mukherjee 1970; Chatterjee and Mukherjee 1977). In view of the general correlation between active genes and their replication in early S phase and vice versa (Goldmann et al. 1984; Holmquist 1989), it has generally been presumed that the early completion of replication by the male X chromosome in polytene cells is causally related to its hyperactivity in relation to dosage compensation (Lucchesi and Manning 1987; Mukherjee 1990). In a recent report, Kar and Mukherjee (1993), using cell fusion to induce premature chromosome condensation (PCC), claimed that the X chromosome in mitotically dividing nuclei of *Drosophila melanogaster* males was also early replicating. On the other hand, Lakhotia and Sinha (1983) had suggested that the faster completion of replication by the hyperactive

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Table 1. Synchrony in replication of autosomes and X chromosome(s) in male and female mitotic cells of *Drosophila nasuta*.

		Number of nuclei with different labelling patterns								
		Autosomes			X chromosome				Y chromosome	
Sex	Tissue	E	B	H	U	E	B	H	U	L
Male	Embryo	4			0	4	0	0	2	2
			2		0	0	2	0	1	1
				9	0	0	0	9	0	9
Female	Embryo	45			0	45	0	0		
			52		0	0	52	0		
				130	2	0	0	128		
Male	Brain	34			0	34	0	0	12	22
			16		0	1	15	0	1	15
				84	1	0	0	83	2	82
Female	Brain	149			3	146	0	0		
			127		1	0	126	0		
				292	17[a]	0	0	275		

NOTE: U, unlabelled; E, only euchromatin regions labelled; B, both hetero- as well as eu-chromatin regions labelled; H, only heterochromatin regions labelled; L, Y chromosome labelled.

[a]Both X chromosomes unlabelled in one metaphase plate; in all other female metaphases in the X-unlabelled category, only one X chromosome was unlabelled, while the other was labelled the same as the autosomes.

X chromosome in male polytene nuclei could be a consequence of certain specific features of the organization of polytene chromosomes rather than being a causal factor for hyperactivity. In the present study, we have directly examined the temporal order of replication of the X chromosome in early embryonic and mitotic larval brain cells of male and female *D. nasuta* using [^{3}H]thymidine labelling. Our results show that in mitotic male cells, the single X chromosome is not early replicating, rather it replicates in complete synchrony with autosomes. In female cells also, the two X chromosomes replicate in synchrony with each other as well as with autosomes.

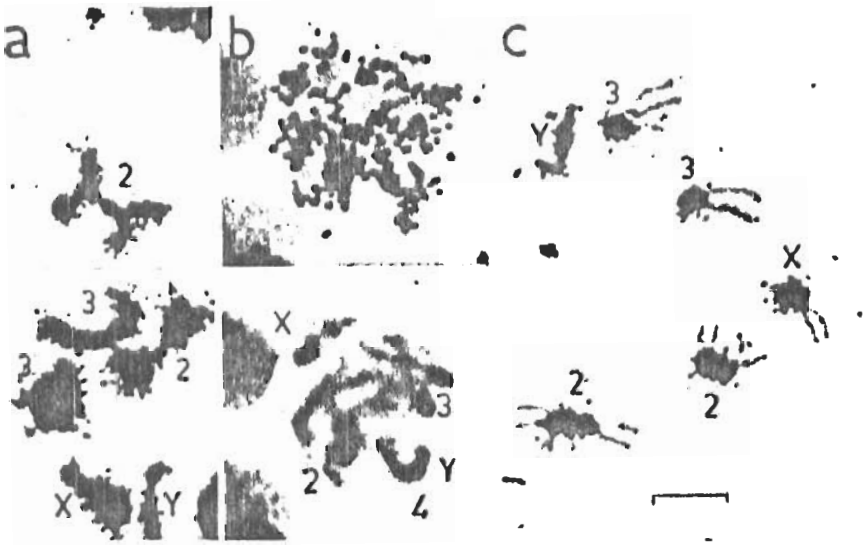
Materials and methods

Flies and larvae of a wild-type strain (Varanasi) of *D. nasuta* were reared on a standard *Drosophila* food medium at 24 ± 1°C. For [^{3}H]thymidine labelling of embryonic cells, embryos 4–5 h old were removed from the food surface by brushing, washed in Poels' salt solution (Lakhotia and Mukherjee 1980), dechorionated with 4% bleaching powder solution, and again washed several times with water followed by a wash with Poels' salt solution. Finally, the embryos were transferred to modified Poels' culture medium (Sinha and Lakhotia 1980) in sterile cavity slides and broken with fine steel needles into 2 to 3 pieces. In order to label the embryonic cells, [^{3}H]thymidine (740 kBq/mL; specific activity 584.6 GBq/mM, Bhabha Atomic Research Centre (BARC), Trombay, India) was added to the medium for 10 min, after which the embryo pieces were repeatedly washed with radioisotope-free medium supplemented with yeast extract (2 mg/mL) and "cold" thymidine (10 μg/mL). The washed embryo pieces were transferred to a fresh cavity slide and chased in the supplemented medium for 15 min to 4 h. Colchicine (1 μg/mL) was added to this medium for the last 15 to 30 min. Finally, air-dried preparations were made as described earlier (Lakhotia and Roy 1981). To label brain ganglia with [^{3}H]thymidine, the ganglia were dissected from late 3rd instar larvae in modified Poels' medium and immediately pulse labelled with [^{3}H]thymidine (740 kBq/mL; specific activity 584.6 GBq/mM, BARC, Trombay, India) for 10 min, followed by washes with radioisotope-free medium supplemented with yeast extract and "cold" thymidine, as for embryos. The pulse-labelled ganglia were chased in the supplemented medium for 15 min to 24 h; when chased for longer than 2 h, colchicine (1 μg/mL) was added to the chase medium for the last 1 h of culture. Air-dried chromosome preparations were made at the end of the chase period, as described earlier (Lakhotia and Kumar 1978). Slides with labelled cells (embryonic or larval brain ganglia) were processed for autoradiography with Ilford L4 nuclear emulsion. Autoradiograms were stained with Giemsa and scored for the pattern of labelling on individual chromosomes of every metaphase plate examined.

Results and discussion

The karyotype of *D. nasuta* permits very easy identification of each individual chromosome owing to the distinctive shape, size, and heterochromatin content of the chromosomes (Lakhotia and Kumar 1978). The metaphase plates in different autoradiographic preparations were identified as male or female on the basis of their sex-chromosome constitution. Since the embryos or larvae were not preselected on the basis of their sex, this sample (Table 1) turned out to be rather biased for female metaphases. It is known from earlier studies (Barigozzi 1968; Steinemann

Fig. 1. Autoradiograms of [^{3}H]thymidine pulse labelled metaphase plates from brain ganglia of male larvae of *D. nasuta*. (*a*) Metaphase with euchromatin regions of all chromosomes labelled (the heterochromatic Y chromosome (Y) is almost unlabelled). (*b*) Metaphase with both hetero- and eu-chromatin regions of all chromosomes labelled. (Giemsa-stained metaphase chromosomes after removal of silver grains from the autoradiogram are shown in the lower half of the figure to help identify each chromosome). (*c*) A metaphase with labelling on the heterochromatin of all chromosomes. Note that in all cases the labelling of the X chromosome (X) is similar to that on the autosomes. Scale bar = 5 μm.



1980; Spradling and Orr-Weaver 1987), that when pulse labelled with [^{3}H]thymidine, chromosomes in metaphase cells of *Drosophila* show three distinct patterns of labelling: (*i*) E-labelled, in which only the euchromatin regions are uniformly labelled, (*ii*) B-labelled, in which both euchromatin and heterochromatin regions are uniformly labelled, and (*iii*) H-labelled, in which the label is restricted to heterochromatin regions only (see Fig. 1). As in other systems, in *Drosophila* cells, the E-labelled metaphases represent early S phase, while the H-labelled metaphases represent the late S phase, and the B-labelled metaphases represent the intervening period (Barigozzi 1968; Steinemann 1980; Spradling and Orr-Weaver 1987). In agreement with this temporal order, in the present study, the H-labelled metaphases appeared first, in the early (15 min) chase samples, followed by the B- and E-labelled types, in that order, in later chase samples. However, in brain ganglia, the H-labelled metaphases persisted in later samples also. In nearly all the labelled metaphases examined from early embryos or brain ganglia, the different autosomes showed remarkable synchrony in the labelling of their euchromatin, both eu- and hetero-chromatin, or heterochromatin regions, and accordingly, the labelled metaphases were classified as E, B, or H type, respectively (Fig. 1; Table 1). The labelling of the X and Y chromosomes (in the case of male cells) or of the two X chromosomes (in the case of female cells) in each of these different types of labelled metaphases was examined to see if any of these chromosomes replicated out of synchrony with reference to the autosomes in the nucleus. The pooled data for different chase samples of the two tissues are presented in Table 1. As for the autosomes, the labelling of the X chromosome(s) was also classified as unlabelled (U), euchromatin labelled (E), both eu- and hetero-chromatin labelled (B), or heterochromatin labelled (H) type, while the Y chromosome was classified as unlabelled (U) or labelled (L) type (Table 1).

The data in Table 1 show that in all the embryonic male metaphases, the labelling of the autosomes and the X chromosome was highly concordant. The mostly heterochromatic and late-replicating submetacentric Y chromosome displayed low labelling of its tips in several metaphases with E- or B-type autosomal and X-chromosomal labelling; the tips of the Y chromosome are known to be dully fluorescent with Hoechst 33258 (see Lakhotia and Kumar 1978). The X-chromosomal and autosomal labelling patterns were concordant in all the female embryonic metaphases examined except for two H-labelled types, where one X chromosome was unlabelled, while the other showed H labelling (Table 1).

In all the 34 E-labelled metaphases from brain ganglia of male larvae, the X chromosome and autosomes were similarly labelled. In 1 of the 16 B-labelled male brain metaphases, the X chromosome was a E-labelled type, while the labelling of the other 15 was concordant. In only 1 of the 84 H-labelled metaphases examined from brain ganglia of male larvae, was the X chromosome unlabelled; in all others the X chromosome, as well as the autosomes, were H-labelled.

In most of the metaphases from brain ganglia of female larvae, the X chromosomes were labelled in complete synchrony with the autosomes; only in rare cases did one of the two X chromosomes appear unlabelled when the other X chromosome was labelled in synchrony with the autosomes. In rare instances one or the other autosome also appeared

unlabelled or differently labelled from the others (details not presented but the overall incidence of such metaphases was comparable to that for the two X chromosomes in female nuclei).

In the more than 700 metaphases examined in preparations of embryonic cells and larval brain ganglia cells in which the autosomes were unlabelled, the sex chromosomes (both the X chromosomes in female and the X and Y chromosomes in male cells) were also unlabelled.

If the single X chromosome in male cells was early replicating, as in larval salivary gland cells, one would expect the single X chromosome to show B- or H-type labelling in many nuclei with E-labelled autosomes and H-type labelling in nuclei with B-labelled autosomes and finally to see the male X chromosome unlabelled in many of the H-labelled metaphases. However, except for the two rare instances in which the X chromosome in male cells was out of step with the autosomes (in one the male X chromosome (U) was "ahead" of the autosomes (H), while in the other, the male X chromosome (E) was "delayed" with reference to the autosomes (B) (see Table 1)), in the nearly 150 other labelled metaphases from males, the X chromosome was always labelled in complete synchrony with the autosomes. Since a certain degree of interchromosomal asynchrony was seen between the two X chromosomes in female cells or, also, within the autosome set, the two instances of asynchrony between the X chromosome and autosomes seen in male nuclei do not appear to have any significance.

In contrast with our present results with *D. nasuta*, Kar and Mukherjee (1993) concluded that the X chromosome in mitotic cells of *D. melanogaster* males was early replicating, as in polytene cells. It is unlikely that these different results are the result of species differences as evidenced by the following two considerations: (*i*) the hemizygous X chromosome in the polytene nuclei of male *D. nasuta* also replicates early (Roy and Lakhotia 1981) and (*ii*) in a parallel study in our laboratory (Sujata Roy, unpublished data) the X chromosome in mitotic cells of *D. melanogaster* males was also found to replicate in synchrony with autosomes. As pointed out earlier (Lakhotia 1993), the conclusion of Kar and Mukherjee (1993) about early completion of the X chromosome in mitotic cells of male *Drosophila* may not be justified, since their experimental design to obtain PCC in *Drosophila* cells had several flaws, and also, the results presented by them were not unequivocal. Compared with the indirect approach of Kar and Mukherjee (1993), our present study directly examined the temporal order of replication of the X chromosome in mitotic cells and the results show unambiguously that neither the single X chromosome in male cells nor either of the two X chromosomes in female cells displayed any kind of asynchrony with respect to replication of autosomes. Thus, we conclude that the single X chromosome in mitotically dividing cells of *Drosophila* males is not replicating earlier than the autosomes. This may appear to be at odds with the well-established, early completion of an endoreplication cycle by the hemizygous X chromosome in salivary gland polytene cells of male larvae (Lakhotia and Mukherjee 1970; Chatterjee and Mukherjee 1977; Lucchesi and Manning 1987). However, Lakhotia and Sinha (1983) suggested that the early completion of replication by the hemizygous X chromosome in polytene nuclei of male larvae was a special consequence of the unique requirements of the polytene structure, and that the early completion of replication was not a prerequisite for an increased rate of transcription of the somatically active X-linked genes in males to achieve dosage compensation. Another aspect of the organization of active replicons also needs to be considered in this context. In mammalian and other cells, adjacent replicons are organized as "replicon clusters" (Hand 1978) that are all activated synchronously at a given part of the S phase and this clustering of replicons generates "replication bands" at the metaphase chromosome level (Holmquist 1989; Craig and Bickmore 1993). Unlike the case in mammalian cells, replicon clusters are not seen in *Drosophila* (Lakhotia and Sinha 1983; Steinemann 1980; Lakhotia and Tiwari 1985), rather, all the active replicon origins along the entire euchromatin or heterochromatin regions appear to be "fired" together, so that the metaphase chromosomes of *Drosophila*, pulse labelled with [^{3}H]thymidine during the S phase, do not display "replication bands" (Holmquist 1989; Raman and Lakhotia 1990). In the absence of asynchronously replicating "replicon-clusters" in *Drosophila* nuclei, all chromosomes would be expected to replicate synchronously except in polytene nuclei, where the special constraints of polyteny result in asynchronous early replication of the hemizygous X chromosome in male nuclei (Lakhotia and Sinha 1983). Thus a synchronously replicating X chromosome in nonpolytene cells of male *Drosophila* is not in conflict with its transcriptional hyperactivity.

References

Barigozzi, C. 1968. DNA replication in eu- and heterochromatin in *Drosophila*. Nucleus (Calcutta), (Suppl.): 138–144.

Chatterjee, R.N., and Mukherjee, A.S. 1977. Chromosomal basis of dosage compensation in *Drosophila*. IX. Cellular autonomy of the faster replication of the X chromosome of haplo-X cells of *D. melanogaster* and synchronous initiation. J. Cell Biol. **74**: 168–180.

Craig, J.M., and Bickmore, W.A. 1993. Chromosome bands — flavours to savour. Bioessays, **15**: 349–354.

Goldmann, M.A., Holmquist, G.P., Gray, M.C., Caston, L.A., and Nag, A. 1984. Replication timing of genes and middle repetitive sequences. Science (Washington, D.C.), **224**: 686–691.

Hand, R. 1978. Eukaryotic DNA: organization of the genome for replication. Cell, **15**: 317–325.

Holmquist, G.P. 1989. Evolution of chromosome bands: Molecular ecology of noncoding DNA. J. Mol. Evol. **28**: 469–486.

Kar, A., and Mukherjee, A.S. 1993. Induction and characterization of premature chromosome condensation in *Drosophila* synkaryons and implications to dosage compensation. Indian J. Exp. Biol. **31**: 210–214.

Kuroda, M.I., Kernan, M.J., Kreber, R., Ganetzky, B., and Baker, B.B. 1991. The *maleless* protein associates with the X chromosome to regulate dosage compensation in Drosophila. Cell, **66**: 935–947.

Lakhotia, S.C. 1993. Is the X-chromosome early replicating in mitotic cells of *Drosophila* males? Indian J. Exp. Biol. **31**: 661–662.

Lakhotia, S.C., and Kumar, M. 1978. Heterochromatin in mitotic chromosomes of *Drosophila nasuta*. Cytobios, **21**: 79–89.

Lakhotia, S.C., and Mukherjee, A.S. 1969. Chromosomal basis of dosage compensation in *Drosophila*. I. Cellular autonomy of hyperactivity of male X-chromosome in salivary glands and sex differentiation. Genet. Res. **14**: 137–150.

Lakhotia, S.C., and Mukherjee, A.S. 1970. Chromosomal basis of dosage compensation in *Drosophila*. III. Early completion of replication by the polytene X-chromosome in male: further evidence and its implications. J. Cell Biol. **47**: 18–33.

Lakhotia, S.C., and Mukherjee, T. 1980. Specific activation of puff 93D of *Drosophila melanogaster* by benzamide and the effect of benzamide treatment on the heat shock induced puffing activity. Chromosoma, **81**: 125–136.

Lakhotia, S.C., and Roy, J.K. 1981. Effects of Hoechst 33258 on condensation patterns of hetero- and euchromatin in mitotic and interphase nuclei of *Drosophila nasuta*. Exp. Cell Res. **132**: 423–431.

Lakhotia, S.C., and Sinha, P. 1983. Replication in *Drosophila* chromosomes. X. Two kinds of active replicons in salivary gland polytene nuclei and their relation to chromosomal replication pattern. Chromosoma, **88**: 265–276.

Lakhotia, S.C., and Tiwari, P.K. 1985. Replication in *Drosophila* chromosomes. XV. Organization of active replicons in brain ganglia and wing imaginal disks of *D. nasuta* larvae. Indian J. Exp. Biol. **23**: 413–420.

Lucchesi, J.C., and Manning, J.E. 1987. Gene dosage compensation in *Drosophila melanogaster*. *In* Molecular genetics of development. *Edited by* J.G. Scandalios. Academic Press, San Diego. pp. 371–429.

Mukherjee, A.S. 1990. Dosage compensation in *Drosophila* and mammals: A review of the past and speculation for the future. *In* Trends in chromosome research. *Edited by* T. Sharma. Springer-Verlag and Narosa Publ House, New Delhi. pp. 90–109.

Mukherjee, A.S., and Beermann, W. 1965. Synthesis of ribonucleic acid by the X chromosomes of *Drosophila melanogaster* and the problem of dosage compensation. Nature (London), **207**: 785–786.

Palmer, M.J., Mergner, V.A., Richman, R., Manning, J.E., Kuroda, M.I., and Lucchesi, J.C. 1993. The *male-specific lethal-one* (*msl-1*) gene of *Drosophila melanogaster* encodes a novel protein that associates with the X chromosome in males. Genetics, **134**: 545–557.

Raman, R., and Lakhotia, S.C. 1990. Comparative aspects of chromosome replication in *Drosophila* and mammals. *In* Trends in chromosome research. *Edited by* T. Sharma. Springer-Verlag and Narosa Publ House, New Delhi. pp. 69–89.

Roy, S., and Lakhotia, S.C. 1981. Replication in *Drosophila* chromosomes. IV. Patterns of chromosomal replication in salivary gland polytene nuclei of *D. nasuta*. Indian J. Exp. Biol. **19**: 797–807.

Sinha, P., and Lakhotia, S.C. 1980. Replication in *Drosophila* chromosomes. V. Polytene chromosome replication after *in vitro* culture of larval salivary glands. Indian J. Exp. Biol. **18**: 1059–1065.

Spradling, A., and Orr-Weaver, T. 1987. Regulation of DNA replication during *Drosophila* development. Annu. Rev. Genet. **21**: 373–403.

Steinemann, M. 1980. Chromosomal replication in *Drosophila virilis*. I. Diploid karyotype of brain cells. Chromosoma, **78**: 211–223.