INDEPENDENT ENDO-REPLICATION CYCLES OF HETERO- AND EU-CHROMATIN IN MITOTIC CHROMOSOMES IN SOMATIC CELLS OF *Drosophila*

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A. CHROMOSOME CYCLES

In typical eukaryotic cells, every mitotic division is preceded by complete doubling of the nuclear DNA for equal segregation to the two daughter cells. During a normal mitotic cycle, complete chromosomal replication is sequentially followed by chromosome condensation, kinetochore separation, nuclear division and finally, the cytokinesis. Premature completion of a cell cycle after partial or complete chromosome replication but prior to the subsequent events leads to increased nuclear DNA content and specific patterns of genome organization depending upon the point at which the cycle is "short-circuited". Thus conditions like polyteny, polynemy, polyploidy and syncitial stage result from curtailment of the basic mitotic cycle at various points.

In different cell types of *Drosophila*, polytene and polyneme type chromosome cycles are seen in which the curtailment occurs prior to kinetochore separation. To avoid ambiguity, it may be noted that these two terms are being used here to denote specific types of nuclear organization as defined by Rieger et al (1). Thus polyteny implies a specific nuclear differentiation in certain cell types of some insects, protozoa and angiosperms represented by the presence of banded polytene chromosomes. The term polynemy is used to designate either the condition of multistranded (polyneme) metaphase chromosomes or the condition of endoreplication cycles giving rise to interphase nuclei with increased DNA content but with no discrete banded chromosomes or chromosome regions.

B. POLYTENE REPLICATION CYCLES IN *Drosophila*

Polytene chromosomes are found in a variety of larval and adult cell types of *Drosophila*. The most well known are those from the larval salivary glands. The replicative organization of polytene chromosomes has almost exclusively been studied in larval salivary glands, the basic features of which are summarized below (also see ref. 2).

In polytene nuclei the basic unineme chromatid fibril replicates repeatedly and the daughter strands remain relatively extended but their precise lateral association results in the well known banding pattern of these chromosomes. It is now well established that the entire length of the basic chromatid fibril of *Drosophila* chromosomes does not replicate equally during polytene replication cycles : at least three, possibly more, different levels of unequal replication are known (3,4). The bulk of the pericentromeric heterochromatin which is enriched in highly repetitive and/or satellite sequences does not replicate during polytenization and remains condensed as the a-heterochromatin in the chromocentre region of polytene nuclei (5-9). The ribosomal cistrons, located within the a-hetero-

chromatin regions of mitotic chromosomes, show another level of unequal replication; these sequences polytenize but for fewer cycles than the bulk of euchromatin, which provides the 3rd level (10,11). The replication cycles of the ribosomal cistrons, located within the body of the nucleolus of polytene cells, are temporally dissociated from the euchromatin replication cycles (12) and also there are evidences to indicate that the different nucleolar organizer regions do not participate equally in polytenization (13). Replication of different euchromatin sequences in polytene nuclei too has been proposed to be unequal (14) since the relative DNA contents of consecutive band and interband regions have been found to differ so that certain bands may have replicated more often than others. Our recent studies using DNA fibre autoradiographic technique have provided some information on this aspect (15, and other unpublished data). In disagreement with the results of another recent DNA fibre autoradiographic study by Steinemann (16), we find at least two major categories of replicon organization in polytene nuclei : one group being characterised by a long replicon size (about 60µm) and a faster rate (1µm/min) of fork migration while the other group has shorter (about 20µm) replicons and a slower rate (0.1µm/min) of fork migration. We have also obtained evidence which suggests that the first group of replicons is active in the early part of a polytene S-period and the second group (shorter and slower replicons) during the later part when heterochromatic chromocentre and certain band regions (17) are replicating. It appears to us that this dual replicon organization is related to the unequal polytenization of different band and interband regions as proposed by Laird (14). This aspect will be discussed in detail elsewhere.

The most significant point that emerges from the above features of polytene replication cycles is that different DNA sequences, even those located on the same chromosome and thereby expected to be part of the same long DNA molecule (18) participate unequally in polytene replication cycles. Obviously, Drosophila genome has the necessary genetic information to generate regulatory signals which identify specific sequences and programme their unequal participation in the consecutive endoreplication cycles which result in the highly polytenized nuclei.

C. CHROMOSOME CYCLES IN BRAIN CELLS OF Drosophila LARVAE

Larval brain ganglia contain a heterogenous mixture of cells in which some divide mitotically and some grow by endoreplication cycles but do not develop a polytene organization; rather they remain polyneme type interphase nuclei. A few true polytene nuclei are also seen in brain ganglia.

It is interesting that in these endoreplicating interphase nuclei also different DNA sequences replicate unequally. The first evidence for unequal endoreplication of the hetero- and euchromatin regions in larval brain cells was provided by the cytophotometric analysis of Berendes and Keyl (19). Their data showed that many of the larval brain cells in D.hydei have more DNA than expected in a 2C/4C cell population but the relative increase in DNA content in hetero- and euchromatin regions of a nucleus were found to be not same, the heterochromatic chromocentre usually showing lesser

increase. This indicates that in larval brain cells, the hetero- and euchromatin may endoreplicate to different levels. It may be noted that unlike in polytene nuclei in which bulk of the heterochromatin probably never replicates (5-9), in larval brain cells the heterochromatin also endoreplicates but usually less often than euchromatin. Moreover, since the euchromatin regions in larval brain cells also do not attain very high levels as in salivary gland polytene cells, the disparity in the relative contents of eu-and heterochromatin in larval brain tissue is much less marked and therefore, all the satellite and/or highly repetitive sequences are identifiable in larval brain tissue (7).

Fluorescence and autoradiographic studies on brain cells of D.nasuta in my laboratory (20) have confirmed Berendes and Keyl's (19) observations. On the basis of these results, the endoreplication cycles of hetero- and euchromatin regions of a nucleus in larval brain appear to be autonomous so that a nucleus may replicate its euchromatin regions for one or more cycles without any immediate DNA synthesis in the heterochromatin; after a few such partial endoreplication cycles, the heterochromatin regions may also replicate for one or more replication cycles. Such partial endoreplication cycles result in polyneme interphase nuclei having varying levels of under- of over-replication of heterochromatin (19,20). In a recent study, Grimm and Kunz (21) have observed disproportionate amounts of rDNA sequences in larval brain cells of certain translocation (Y-autosome) stocks of D.hydei. Since these authors have considered the larval brain tissue to be "diploid", they believe "this rDNA increase can not be the result of regulatory mechanisms which act during endomitotic DNA replication". However, in my view the observed disproportionate rDNA replication is indeed related to the endoreplication cycles taking place in larval brain cells. It is likely that the increased number of rDNA sequences found a particular genotype, in comparison to the wild type or other genotypes, is related to alterations in the endoreplication programme of specific sequences brought about by chromosome rearrangement (see Section D).

The mitotic cells in larval brain always show diploid chromosome number inspite of the high incidence of endoreplicating cells in the tissue. If the endoreplicating cells occasionally switch over to a mitotic cycle, they would be expected to generate either polyploid metaphases with unineme chromatids or diploid metaphases with polyneme chromatids. The lateral strandedness of metaphase chromosomes in brain tissue of Drosophila larvae has been a matter of debate. In view of the normal diploid chromosome number in all mitotic cells and the presence of all satellite sequences in larval brain ganglia, and the overwhelming general evidnece for uninemy of eukaryotic chromosomes, the brain tissue of Drosophila larvae has usually been considered to consist largely of typical 2C/4C cells (7,21,22). However, in view of the above noted evidences for the occurrence of endoreplication cycles, it appears erroneous to consider the larval brain tissue to be predominantly diploid. As mentioned earlier, the occurrence of all satellite and repetitive sequences in larval brain ganglia does not demand a diploid condition since their presence is to be expected with the pattern of endoreplication cycles occuring in these cells. Thus the possibility of mitotic chromosomes in this organ being polyneme can not be

ruled out.

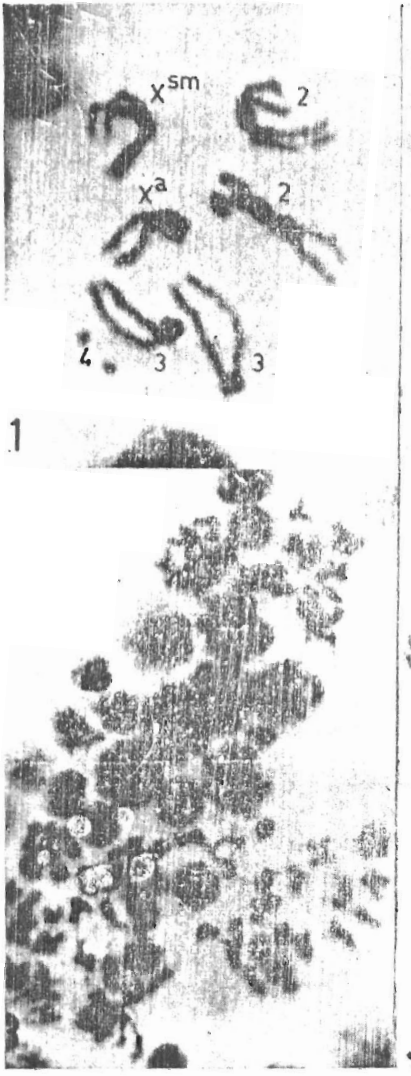
Earlier cytophotometric studies (23) suggested an increase in the DNA content in metaphase cells in 3rd instar larval brain over those in the 1st instar larval brain and this was implied to indicate increase in the lateral strandedness with increasing larval age (23). These results, however, have not been confirmed in a later study (24). We have been studying the strandedness of metaphase chromosomes in brain cells of D.nasuta larvae for the past few years (25,26) by applying the very sensitive bromodeoxyuridine -Giemsa staining and ^{3}H-thymidine autoradiography techniques. Our results have favoured the interpretation that while the undifferentiated early embryonic cells of Drosophila have unineme chromosomes, in late larval brain cells, the mitotic cells have multi-stranded chromosomes. This aspect has been discussed in detail elsewhere (27,28).

On the basis of above, we believe that in brain tissue of Drosophila larvae, repeated endoreplication cycles may be periodically interrupted by chromosome condensation and mitotic division cycle so that polyneme interphase nuclei give rise to "diploid" metaphases with polyneme chromosomes. In this context, it may be asked if the endoreplicating cells which decide to switch over to a mitotic division are only those in which the hetero- and euchromatin regions both have replicated immediately prior to mitosis or other cells may also come to mitosis ? I will present here some results which suggest that the endoreplicated interphase nuclei in which the heterochromatin and euchromatin regions have replicated in independent cycles, and possibly unequally, also enter mitosis.

These studies have been done with brain ganglia from late 3rd instar larvae of D.nasuta. The mitotic karyotype (2n=8) in this species includes 3 pairs of large chromsomes (acrocentric or sub-metacentric X, metacentric 2nd and acrocentric 3rd) and a pair of small dot-like 4th chromosomes; the Y-chromosome in male is a medium-sized sub-metacentric (28,29). All chromosomes, otherthan the tiny 4th, carry prominent pericentromeric heterochromatin blocks (see fig. 1).

The excised larval brain ganglia were cultured in vitro (30) for labelling with ^{3}H-thymidine. In one set, freshly excised ganglia were pulse labelled with ^{3}H-thymidine (50µCi/ml; sp.act. 15.8 Ci/mM, BARC) for 30 min, immediately fixed in fresh 1:3 aceto-methanol and air-dry preparations (29) were made. In a second set, freshly excised ganglia were cultured in the presence of ^{3}H-thymidine (2µCi/ml) and colchicine (0.5µg/ml) for 24h or 48h. In a 3rd set, the ganglia were cultured in the presence of ^{3}H-thymidine (2µCi/ml) for a period of 24h of which the first 19h were without and the last 5h were with colchicine (0.5µg/ml).

Since the replication cycles of different cells in brain ganglia of normally growing 3rd instar larva are highly asynchronous, in another series of experiments, an attempt was made to synchronize their replication cycles by feeding mid 3rd instar larvae on 5-fluorodeoxyuridine (FdU) supplemented medium (200µg/g food). It has been shown that after 24h FdU-feeding, the replication cycles in nearly all salivary gland polytene nuclei are blocked at the beginning of the S-period (31,32). For the present study also, in the initial experiments the larvae were fed for 24h; results suggested that the



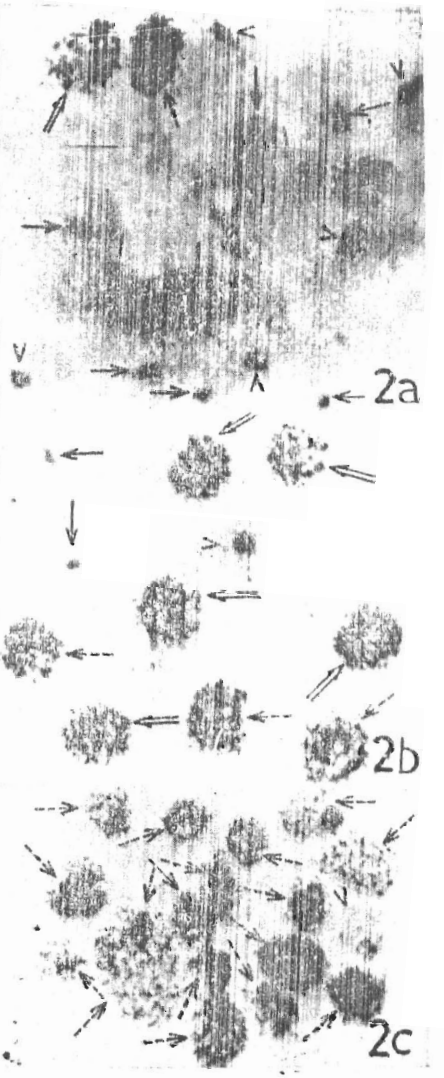


Fig. 1. Metaphase chromosomes from late 3rd instar larval brain ganglia of D.nasuta. Note the dark stained heterochromatin regions of all the larger chromosomes. x 2800.

Fig. 2a-c. Patterns of ^{3}H-thymidine labelling of larval brain interphase nuclei : a unsynchronized ganglia pulse labelled; b 7h in vivo FdU-synchronized ganglia pulse labelled; c FdU-synchronized ganglia chronically labelled for 24h. Unlabelled —>; chromocentre labelled >; euchromatin labelled ⟹; both labelled ⟹. x 1000.

Fig. 3. A cluster of metaphases in brain ganglia exposed to colchicine in vitro for 24h. x 1000.

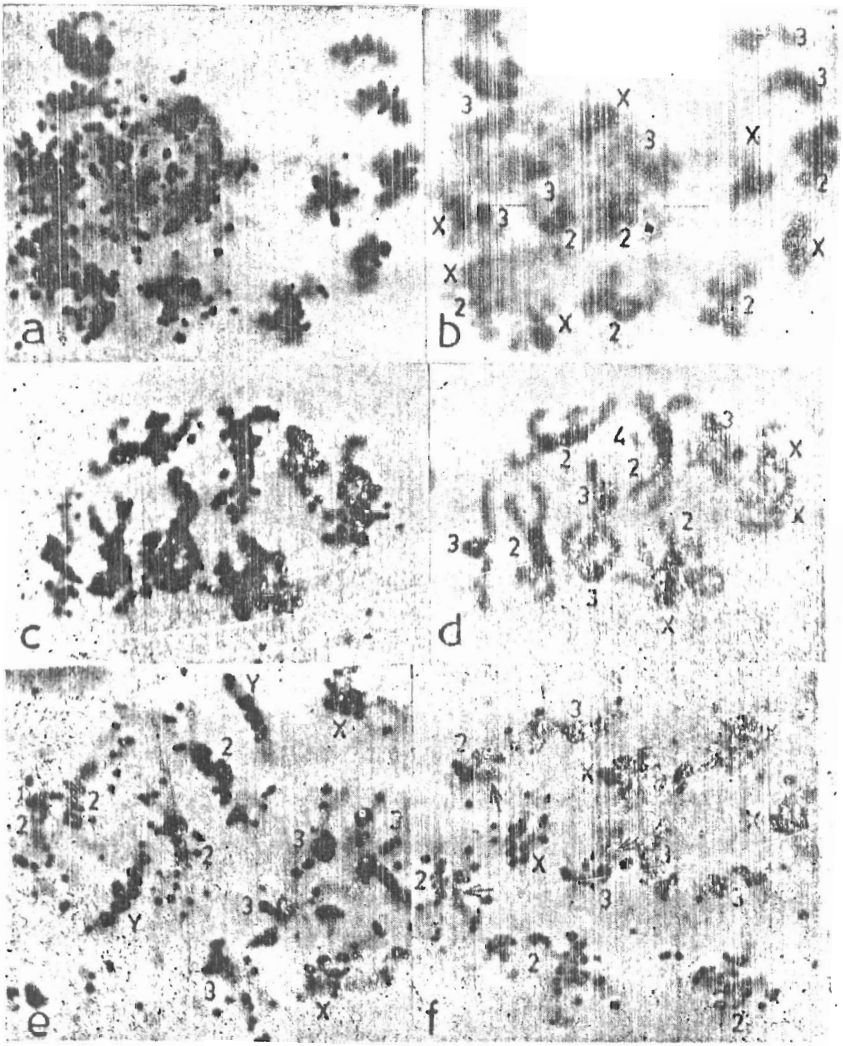


Fig. 4. Metaphases in unsynchronized brain ganglia labelled chronically with ^{3}H-thymidine for 48h (a-d) or 24h (e-f). a A 4N prometaphase and a 2N metaphase with only the heterochromatin regions labelled, b the same plates after removal of silver grains; c a 4N metaphase with heavy labelling of heterochromatin of both chromatids of all chromosomes but with only one chromatid of each chromosome labelled in the euchromatin regions, d same plate after degraining; e a 4N metaphase with labelling mainly on the heterochromatin regions of all chromosomes; f another 4N metaphase with predominant labelling of heterochromatin regions but in some chromosomes, one of the chromatids is distinctly unlabelled (→). x 2800.

replicating nuclei in brain ganglia are being synchronized; however, the mitotic chromosomes in these 24h FdU-fed larvae showed some damage and therefore, in later experiments shorter durations of FdU treatment were applied. It was found that a 7h FdU-feeding also induces as good synchrony in replicating brain cells as a 24h feeding period but with little chromosome damage and therefore, in the experiments reported here, mid 3rd instar larvae of D.nasuta were fed on FdU-food for 7h only. After this synchronization period, the brain ganglia were excised and one set was immediately pulse labelled with ^{3}H-thymidine (50µCi/ml) for 30 min and fixed; a 2nd set of ganglia was cultured in vitro for 24h in the continued presence of ^{3}H-thymidine (2µCi/ml) and colchicine (0.5µg/ml). In a 3rd set, the excised ganglia were first labelled with ^{3}H-thymidine for 19h after which colchicine was added to the same medium and 5h later the ganglia were harvested.

In each of the above cases, after the period of labelling air-dried chromosome preparations were made and autoradiographed in the usual manner. For each labelling schedule at least 6 brain ganglia were used. The availability of ^{3}H-thymidine in the medium after the 24h or 48h labelling period was ascertained by culturing fresh ganglia in the used culture medium : in each case it was found that the fresh ganglia are as well labelled in the used medium as in the fresh medium and this assured that the label was available in the medium for the entire duration.

The autoradiograms were scored for the patterns of labelling on interphase and metaphase nuclei. The data on the labelling patterns of interphase nuclei in 0h pulse and 24h chronic (with colchicine during the last 5h) labelled ganglia from unsynchronized and in vivo FdU-synchronized larvae are presented in Table 1. In all cases, 65 to 85% interphase nuclei (larger as well as smaller sized) remain unlabelled while among the labelled nuclei, 3 categories are seen : i)only the heterochromatic chromocentre labelled; ii) only the euchromatic non-chromocentre region labelled, and iii) both regions labelled (fig. 2). It is seen that compared to the 0h pulse labelled samples, the frequencies of completely labelled nuclei increase while those of euchromatin labelled nuclei decreases in 24h chronic labelled ganglia both in the unsynchronized and synchronized series (see Table 1). This shift is more pronounced in the FdU-synchronized ganglia since in them the initial frequency of euchromatin labelled nuclei is much higher which indicated that as in the case of salivary gland polytene nuclei (31,32), in brain ganglia also FdU has blocked replicating nuclei at the beginning of their S-period. In this context, the persistence of about 8% nuclei with ^{3}H-thymidine incorporation restricted to the heterochromatin region in the synchronized brain cells (0h) is significant and appears to be related to the earlier discussed autonomy of endoreplication cycles of eu- and heterochromatin regions of a nucleus; prior to FdU-block, these nuclei may have been programmed or engaged in endoreplication of heterochromatin and upon release from the block by ^{3}H-thymidine, they resumed DNA synthesis in heterochromatin. In unsynchronized as well as synchronized ganglia labelled with ^{3}H-thymidine for 24h, about 9-10% nuclei still show heterochromatin labelling (Table 1). In an earlier study (20) also we found about 5% nuclei showing DNA synthesis only in the heterochromatin region during a 66h long labelling period. A consideration of the labelling patterns of

metaphases in different samples (table 2) suggests that the heterochromatin labelled interphase cells do not belong to a static population; rather many of these cells enter mitosis since the proportion of metaphases with ^{3}H-thymidine labelling in heterochromatin regions only is high in all the samples analysed (see table 2). Thus to maintain the pool of 5 to 10% heterochromatin labelled interphase nuclei at the end of long chronic labelling periods, fresh cells must continue to enter heterochromatin replication cycles during the labelling period.

Table 1. PATTERNS OF AUTORADIOGRAPHIC LABELLING OF INTERPHASE NUCLEI IN BRAIN GANGLIA LABELLED IN VITRO WITH ^{3}H-THYMIDINE

Culture	Labelling Period	Total Nuclei	% Different Labelling Patterns*		
			Eu-Labelled	Hetero-Labelled	All Labelled
Unsynchronized	30min pulse (0h sample)	1831	7.9	13.8	1.4
- do -	24h chronic	2235	0.9	9.3	6.4
Synchronized	30min pulse (0h sample)	2811	24.2	8.3	2.3
-do -	24h chronic	1734	0.5	9.8	19.9

* the remaining nuclei are unlabelled in each sample

TABLE 2. PATTERNS OF AUTORADIOGRAPHIC LABELLING OF 2N METAPHASES IN BRAIN GANGLIA CHRONICALLY LABELLED IN VITRO WITH ^{3}H-THYMIDINE

Culture	Labelling Period	Colchicine Period	Total Metaphases	Frequency (%)		
				Unlabelled	All Lab.	Hetero-Labelled
Unsynchronized	0-24h	0-24h	883	35.8	24.5	39.7
- do -	0-24h	19-24h	529	18.9	49.1	31.9
- do -	0-48h	0-48h	760	34.3	18.9	46.7
Synchronized	0-24h	0-24h	158	9.5	68.3	22.1
- do -	0-24h	19-24h	369	6.0	77.2	16.8

For analysing the replication patterns of cells progressing into mitosis, long periods of labelling (24 and 48h) have been used and also colchicine was either given for the entire period or only during the last 5h of labelling. In the case of typical mitotically cycling cell populations in which during every S-period, euchromatin replication in early and mid-S is chronologically followed by heterochromatin replication in late S, specific predictions can be made regarding the labelling patterns observable on metaphases in such experiments. In an asynchronously proliferating population of cells labelled for long periods (longer than average cell cycle duration) with ^{3}H-thymidine in the presence of colchicine, the proportion of unlabelled and only heterochromatin labelled metapha-

ses should decline with increasing durations of labelling and colchicine exposure since during the longer time span, more and more early S and G_1 cells would have traversed through the interphase and get blocked upon reaching metaphase stage; thus completely labelled metaphases would continuously dilute the pool of earlier blocked unlabelled and heterochromatin labelled metaphases. On the other hand in those chronically labelled cultures where colchicine is added only during the last few hours of culture, the proportion of unlabelled and heterochromatin labelled metaphases should be very low if the average cell cycle duration is shorter than the ^{3}H-thymidine labelling period. It may be notedthat the 24h ^{3}H-thymidine labelling period employed in this study is longer than the estimates of the average cell cycle duration in brain ganglia of Drosophila (22,30,33).

Results of the present study, shown in table 2, do not conform to the above expectations. Before considering the results , it may also be noted that under the in vitro culture conditions used in these studies, the progression of cells to mitosis is not disturbed since the mitotic index has been seen to be progressively higher with increasing length of colchicine exposure (data not presented, but see fig. 3); besides the frequency of 4N or even 8N metaphases (arising due to progression of some of the arrested metaphases to 2nd or 3rd mitotic cycle) has also been found to increase with increasing colchicine exposure period. Thus the present results are not distorted due to impaired mitotic progression rates. To ensure that the observed labelling patterns are those of the 1st cycle metaphases, the 4N metaphases present in different samples have not been included in the data presented in table 2. In the unsynchronized 24h labelled samples, although the frequency of unlabelled metaphases in ganglia exposed to colchicine only for the last 5h is nearly half that in the 24h colchicine exposed ganlgia, the frequency of heterochromatin labelled metaphases is only slightly less in the 5h colchicine (31.9%) than in the 24h colchicine (39.7%) sample (see Table 2). The higher frequency (46%) of heterochromatin labelled metaphases in ganglia labelled with ^{3}H-thymidine for 48h in the presence of colchicine is also contrary to the expectations based upon typical mitotic cell cycles. The decline in the frequency of unlabelled and heterochromatin labelled metaphases in the FdU synchronized series compared to those in the unsynchronized series appears to reflect the overall mitotic synchrony induced by the FdU treatment. However, in these 24h ^{3}H-thymidine labelled synchronized ganglia also, the frequency of heterochromatin labelled metaphases in the 5h colchicine exposure series (16.8%) is not much reduced compared to that seen in the 24h colchicine exposure series (22.1%, table 2).

These observations on the metaphase labelling patterns when considered in conjunction with the earlier (20) demonstrated autonomy of hetero- and eu-chromatin endoreplication cycles in larval brain cells and the interphase labelling data presented here, lead us to believe that in the larval brain tissue a cell entering mitosis may have had replicated only the heterochromatin regions during the S-period immediately preceding mitosis. It is possible that during the earlier endoreplication phase, the euchromatin regions of a nucleus replicated a few times more than the heterochromatin so that the nucleus at this stage has a disproportionately lower heterochromatin content than prior to endoreplication cycles. When this cell

has to enter mitosis, it may need to replicate only its heterochromatin regions. In previous studies (34,35) evidence was obtained for more frequent replication of euchromatin during endoreplication cycles progressing in early stages of larval development.

Since brain cells which have had replicated eu- and heterochromatin regions in independent endoreplication cycles have been seen to enter mitosis with multi-stranded chromosomes, the possibility that as in polytene chromosomes, in the polyneme metaphase chromosomes also the number of lateral strands in the eu- and heterochromatin regions is unequal, needs consideration. This situation would arise if endoreplicated cells having disproportionate levels of hetero- and euchromatin were to be triggered to enter mitosis. Evidence in favour of these events have been obtained in the results of above mentioned experiments. As mentioned earlier, in the preparations of brain ganglia labelled continuously with ^{3}H-thymidine for 24 or 48h in the presence of colchicine, 4N metaphases are also present. In the unsynchronized set, about 6% of all metaphases in the 24h sample and about 14% in the 48h sample are tetraploid. In the synchronized 24h set, the frequency of 4N metaphases is higher (35%) presumably because of the FdU induced synchronous progression of cells to mitosis. As expected all the 4N metaphases are labelled with ^{3}H-thymidine. However, it is very significant that about 10 to 15% of these 4N metaphases show labelling of only the heterochromatin regions (fig. 4). Since 4N metaphases are never seen after a shorter colchicine exposure (23,27), those seen after the longer colchicine treatment must be the arrested 2N metaphase cells entering a second mitotic cycle. Thus all the 4N metaphase cells seen in this study must have traversed through at least one S-period in the presence of ^{3}H-thymidine; typically, these 4N metaphases then should show uniform labelling of eu- as well as heterochromatin regions. Labelling of only the heterochromatin regions in some of the 4N cells shows that the euchromatin regions of different chromosomes did not replicate in the S-period immediately preceding the 4N stage. This implies that either these 4N metaphases have over-replicated heterochromatin or that at the 2N stage, the metaphase chromosomes contained under-replicated heterochromatin regions. In either case, these results make it possible that metaphase chromosomes with unequal number of lateral strands in hetero- and euchromatin regions may occur in larval brain ganglia. Whether the completely labelled metaphases may also have unequal lateral multiplicity of certain regions can not be ascertained from present results. We are now examining this aspect by cytophotometric methods.

Results comparable to the above have also been obtained in parallel experiments (data not presented here) using bromodeoxyuridine or ^{3}H-bromodeoxyuridine incorporation and Giemsa staining and autoradiography.

Other unusual types of autoradiographic labelling have also been seen in the 2N and 4N metaphases in these preparations of brain ganglia chronically labelled with ^{3}H-thymidine for 24 or 48h. In all samples, in 10 to 20% of the heterochromatin labelled 2N metaphases some chromatids of different chromosomes or both chromatids of some chromosomes were not labelled. In a few of the 4N metaphases, both chromatids of all chromosomes were labelled in heterochromatin regions but in the euchromatin regions, labelling was present only on one of the two sister chromatids of all or some chromosomes (see fig. 4). Likewise, in several other 4N metaphases,

while all sister euchromatin regions were labelled, several heterochromatin segments were not labelled. Significance of such labelling patterns is not immediately clear but their presence is indicative of rather unusual patterns of replication and segregation of the label to daughter chromatids in these polyneme chromosomes.

Some other features of metaphase chromosome labelling in brain ganglia of D.nasuta larvae may also be noted here. During our studies it has often been noted that if ganglia are pulse labelled for 20 to 30 min with ^{3}H-thymidine and immediately fixed, a few of the metaphases present in these preparations show distinct labelling of heterochromatin regions of all chromosomes; this suggests a near absence of G_2 phase in these cells; besides about 10 to 15% of metaphase cells have been seen to complete 2 mitotic cycles within 3 to 5h as evidenced by the patterns of BrdU-Giemsa staining of chromosomes (30) and also by the appearance of a few 4N metaphases in ganglia exposed to colchicine for 4-5h. These observations may indicate a relatively brief mitotic cell cycle duration in brain ganglia of D.nasuta larvae. However, other observations suggest a much longer mitotic cycle duration in these cells ; in the present study it has been seen that even after 19h ^{3}H-thymidine labelling followed by 5h colchicine (and ^{3}H-thymidine), about 19% of the metaphases remain unlabelled; in an earlier study (35) about 12% metaphases were seen to remain unlabelled after 66h labelling (with colchicine for the last 1h) of brain ganglia with ^{3}H-thymidine. These observations suggest an unusually long G_2 period in these cells. Equally intriguing is the observation that when brain ganglia of D.nasuta are pulse labelled with ^{3}H-thymidine, chased in radio-isotope free medium and exposed to colchicine only for the last 1-2h, metaphases with both chromatids labelled either in hetero- or euchromatin or both regions continue to be seen in high frequencies even as late as 60h after the pulse (35, and other unpublished data). Simplest interpretation of all these observations would appear to be that in the endoreplicating mitotic cell population in brain ganglia of D.nasuta larvae the "G_2" period is highly variable. As discussed elsewhere (27), the persistence of labelling of both chromatids of all or some chromosomes in the later chase samples is more likely to be related to these chromosomes being polyneme.

There have been relatively fewer earlier attempts to study the cell cycle patterns in brain ganglia of Drosophila larvae. Steinemann (22) has estimated the cell cycle parameters in brain ganglia of D.virilis larvae on the assumptions that the brain cells are diploid and are an exponentially growing population of cells. On these assumptions, Steinemann estimates the cell cycle duration to be about 23h, S about 12h and G_2 about 7h. Our observations on brain ganglia are not in agreement with Steinemann's (22) conclusions. Species differences could account for some but not all the differences between the two sets of observations. It is, however, to be noted that Steinemann (22) has totally ignored endoreplication cycles in larval brain cells although the photomicrographs of interphase nuclei presented in his fig. 2 distinctly show the presence of very large and smaller nuclei which suggests that in D.virilis brain ganglia too endoreplication cycles occur as in D.hydei (19), D.melanogaster (23) and D.nasuta (20). It also appears likely that the ice treatment employed by Steinemann (22) to stop the pulse before chase in the radio-isotope free medium, alters the progression of different cells

to endo- or mitotic replication cycles. Moreover, pulse labelling studies alone do not provide information about the endoreplicating cells and their progression to mitosis. Thus Steinemann's (22) results are not necessarily in conflict with the present observations, although the interpretations in that study appear to be too simplified and based on incorrect assumptions of larval brain cells being diploid and exponentially progressing.

To summarise the above, the following proposal is made to satisfactorily explain the varied observations made on the replication patterns in brain cells of D.nasuta larvae : in these cells the endoreplication and mitotic cycles are not mutually exclusive; a transition probability (analogous to the transition probability of entry of G_o or G_1 cells into S-period in other typical mitotically cycling cells, see ref. 36) determines the passage of an endoreplicating cell to mitotic division cycle with polyneme chromosomes; thus an endoreplicated cell may immediately divide after its last DNA synthesis period or may remain quiscent for a longer time before it is triggered to enter mitosis in a probabilistic fashion. During the intermitotic period the nucleus may endoreplicate its eu- and hetero-chromatin regions in independent and possibly unequal cycles.

D. GENETIC AND DEVELOPMENTAL IMPLICATIONS OF ENDOREPLICATION COUPLED MITOTIC CELL CYCLES

Role of heterochromatin in cellular activity is not known. Yet the quality and quantity of heterochromatin in the genome is well known to influence the phenotype in various ways in Drosophila and other organisms. It is also significant that the satellite sequence composition in different cell types of Drosophila larvae and adult varies in a characteristic manner (37). Apparently, this variability is generated by specific patterns of replication.

Unequal participation of different DNA sequences in polytene replication cycles in Drosophila has been demonstrated in several cases and implied in others (see Section B). Our results (Section C) on the larval brain cells have raised the possibility of comparable unequal replication of different sequences in mitotic chromosomes as well. In the simplest terms, we may presume that the same mechanisms which cause and regulate differential replication of specific sequences in polytene chromosomes, are operative in mitotically active polyname somatic cells also. It is very likely that a multistranded organization of mitotic nuclei provides the basis for generation and propagation of cells having different relative amounts of satellite and other sequences in a cell and tissue-specific manner. Non-polytene cells in adult tissues of Drosophila have been found to have varying amounts of different satellite sequences (37). Moreover, Spradling and Mahowald (38) have shown amplification of chorion genes in endoreplicated follicle cells in ovary of adult Drosophila. This later observation also raises the possibility that selective gene amplification of highly active loci in specific cell types of Drosophila may be more common than hitherto believed (38). It is significant that the so far demonstrated instances of selective gene amplification in Dipteran cells have been found either in highly polytene cells ("DNA puffs" in Sciara, ref. 39) or in endoreplicated ovarian follicle cells of Drosophila (38); the possible unequal polytenization of different bands in salivary glands of Drosophila larvae (14,40) may also be related to comparable gene amplification processes. Therefore, it may appear that endoreplication cycles

are associated with the process of selective gene amplification, at least in Drosophila. Our demonstration of widespread occurrence of endoreplication cycles even in the mitotically active somatic cells of Drosophila thus becomes significant from the viewpoint of cell differentiation.

An interesting but little understood process related to heterochromatin is the phenomenon of position-effect variegation. In Drosophila and several other eukaryotes, it is known that when a chromosomal rearrangement relocates a euchromatic region within or very close to heterochromatin, the expression of genes in the relocated position becomes variable and this results in a variegated phenotype if the relocated segment bears a dominant wild type gene and the unaltered homolog carries its recessive allele. Extensive genetic and developmental studies have been carried out on position effect variegation in Drosophila and these have been reviewed several times recently to which reference may be made for details (41, 42). However, inspite of these extensive studies the basis for the variegated expression of genes transposed closer to heterochromatin is not understood. Nevertheless, it is generally presumed that the activity of the euchromatic locus when placed in proximity to heterochromatin is inhibited in some but not all cells and this results in the variegated expression. Evidences show that the variegating gene is not physically lost from the cells showing mutant phenotype but its activity is altered (43). An important feature of variegation is its clonal expression, i.e., in a clone all cells will either have the relocated gene in inactive state or active state; moreover, the 'decision' regarding activity or inactivity of the transposed locus is made long before the gene has to actually express itself. Thus once a 'decision' for inactivation of the relocated locus has been made in a cell, all its progeny continue to show inactivity of the locus (see ref. 41,42 for further details).

Due to increased cytological resolution offered by polytene chromosomes, a cytological approach has also been made to study this phenomenon. Without going into details of the various observations on this aspect, it may be briefly stated that the euchromatic segments transposed to heterochromatin generally show a variable degree of under-replication and "heterochromatinization" (40,44-48). The altered nucleic acid metabolism of chromosomes (44) or variable under-replication of euchromatin loci transposed to heterochromatin as seen in polytene nuclei may provide a basis for the variegated phenotype since depending upon the extent of under-replication of the relocated locus, its transcriptional activity may be more or less (49). However, in a recent study, Henikoff (50) has in situ hybridised cloned DNA sequences to a variegating locus in polytene nuclei and has found that the level of polyteny is not greatly affected at the inactive gene site and thus he has suggested that the variable ability of a gene to be transcriptionally induced is not dependent upon its gene dosages. However, as also noted by Henikoff (50), in situ hybridization would not detected the differences in polyteny as precisely as cytophotometry. In this context, another observation by Cowell and Hartmann-Goldstein (40) is interesting. They found by microdensitometry of polytene nuclei that while the transposed euchromatin region immediately next to heterochromatin breakpoint contains less DNA than the normal homolog, an adja-

cent intercalary heterochromatin site on the transposed chromosome has more DNA than the homologous site on the normal chromosome. We have also observed (A.Mishra and Lakhotia, unpublished) that in polytene nuclei of D.melanogaster larvae carrying B^{M1} inversion in the X-chromosomes (for details of the inversion, see ref. 51), the replication programme of certain bands away from the heterochromatin relocation point is altered in a characteristic manner although some bands closer to the breakpoint are not so much affected. Viewed in the light of these results, Henikoff's (50) data do not eliminate the possibility that while the replicative programme of the coding sequences has remained relatively unaltered in his material, that of certain other neighbouring sequences does get modified in the rearranged chromosome and which in turn may influence the inducibility of transcription at the locus in the transposed chromosome.

Taken together these observations in salivary gland polytene chromosomes generally support the view held by Schultz (44,49) that the variegated expression of a relocated gene is related to its altered endoreplication programme. Till now it has generally been implied that the altered replication programme of a relocated gene may be a phenomenon restricted to non-proliferating polytene type nuclei. The proliferating mitotic cells, whose terminal progeny contributed to the bulk of adult Drosophila body, have been believed to be diploid and therefore, alterations of the kind seen in polytene replication cycles could not be visualized to be the basis for position effect variegation in imaginal cells (see ref. 42). However, the view proposed here that mitotically proliferating cell in Drosophila may also endoreplicate makes it possible to think of a unified basis for position effect variegation in all cell types of Drosophila. In another study (52) undifferentiated wing imaginal disk cells in 3rd instar larvae of D.nasuta also have been seen to be engaged in endoreplication cycles with disproportionate replication of hetero- and euchromatin. In a more recent study (53) we have obtained evidence for such endoreplication cycles in wing imaginal disks even during early larval stages. Extrapolating the results obtained with larval brain cells, it would appear that the mitotic chromosomes in imaginal disk cells are also polyneme.

In view of the above discussion, the following proposal is made in relation to position effect variegation : In majority of somatic cells of Drosophila endoreplication cycles occur with or without interspersion of mitotic division. During these endoreplication cycles, various levels of under- or over-replication of specific sequences occur in a tissue specific manner; in instances of position effect variegation, these replication cycles are variably modified under the influence of neighbouring sequences and this leads to the variegated expression of the affected gene in different cells because of differences in the quantity and quality either of the coding sequences or of other associated sequences or both. The interspersion of mitotic divisions with endoreplication cycles makes it possible that a particular level of under- or over-replication of certain sequences can be clonally propagated. This can satisfactorily explain the clonal initiation of the variegated effect. The modifying effects of extra heterochromatin, developmental temperature and other factors on the extent of variegation (41,42) may operate through modulation of the endoreplication cycles.

Recent results of Grimm and Kunz (21) on the altered levels of rDNA sequences in brain ganglia of D.hydei larvae carrying certain Y-autosome translocation may thus be related to position effect variegation such that compared to the degree of under-replication of rDNA sequences in the brain cells of wild type larvae, the specific Y-autosome translocation causes the rDNA sequences to escape under-replication during the endoreplication cycles. Earlier observations by Baker (54) on the altered levels of rRNA content in certain X-chromosomal inversions of D.melanogaster which relocate the nucleolar organizer regions from its normal heterochromatin site to the vicinity of euchromatin, may also be related to the changed programme of replication cycles of rDNA in the rearranged position.

If the above proposed mechanism of position effect variegation in Drosophila is valid in its essential principles, the molecular basis of the similar phenomenon associated with the inactive X-chromosome in female mammals (55) would be basically different. The apparent similarity in the phenotypic effects of position effect variegation in Drosophila and mammals need not imply a common basic cause. While in mammals this phenomenon is associated with facultatively heterochromatinized (56) X-chromosome, in Drosophila and several other organisms this phenomenon is related to rearrangements involving constitutive heterochromatin. The inactivation of one X in female mammals has been suggested to be due to specific patterns of methylation of cytosine (57) while in Drosophila genome methyl-cytosine is absent (A. Riggs, personal communication) and thus the "spreading" of inactivity to the relocated euchromatic segments may be due to different causes in the two instances. It remains to be seen if chromosomal rearrangements involving constitutive heterochromatin in mammals also cause position effect variegation.

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