Replication in *Drosophila* Chromosomes: Part XV—Organisation of Active Replicons in Brain Ganglia and Wing Imaginal Disks of *D. nasuta* Larvae

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The organization of active replicons in brain ganglia and wing imaginal disks of late third instar larvae of *D. nasuta* was compared by DNA fiber autoradiography following the 'step down' protocol of 'hot' (15, 30 or 60 min) and 'warm' (50 min) ^{3}H-thymidine pulses. Two distinct categories of active replicons were identified in brain ganglia as well as in wing imaginal disks. The two types differed from each other primarily in their rates of fork migration (0.44-0.52 μm/min, for type I and 0.07-0.08 μm/min, for type II) and also in the overall disposition of arrays of labelled tracks. The type I arrays usually contained several tandemly aligned 'hot' tracks each with flanking 'warm' tails. On the other hand, the type II arrays were often in irregular groups and each array contained fewer dense tracks of relatively uniform and smaller (less than 7 μm) size which were frequently not flanked by warm tails. The modal replicon sizes of the type I and II replicons, determined from 15 and 60 min pulse preparations respectively, in brain ganglia (22.8 and 18.3 μm, respectively) and imaginal disks (19.5 and 18.5 μm, respectively) were similar.

Among the different tissues of *Drosophila* larvae in which active nuclear DNA replication takes place, the brain ganglia and the various imaginal disks comprise a group distinct from the polytene cell types. Some or many of their cells divide mitotically and therefore, these tissues are considered to be diploid cell populations[1-4]. Unlike the extensive information available on polytene nuclei, particularly of larval salivary glands, very little is known of the replicative organisation in the brain ganglia and imaginal disk cells. There has been no cytological analysis of the mitotic cell cycle in imaginal disks although an estimate of mitotic cell cycle in imaginal disks has been made on the basis of direct cell counts or by clonal analysis[5-7]. In the case of larval brain ganglia attempts have been made by several workers to study the cell cycle[8-11]. However, these studies have failed to provide a clear information about the duration of S-period or the generation time in these mitotic cells[10,11]. On the other hand, some studies have indicated that the brain ganglia in *Drosophila* larvae are comprised a heterogenous population of cells several of which may endoreplicate their hetero- and euchromatin components unequally in independent cycles[10-13]. The different cells in an imaginal disk are not of as diverse types as in the brain ganglia, although it has been suggested that hetero- and euchromatin regions in the imaginal disk cells may also endoreplicate unequally[14]. The satellite DNA composition has also been found to vary in different cell types of *Drosophila* larvae and adults[15], presumably as a result of differential replication. In view of these observations, it is of great interest to compare the replicative organisation of nuclear DNA in these cell types. Continuing our studies on replication in different cell types of *Drosophila*, in the present work the organisation of active replicons in cells of brain ganglia and imaginal disks of third instar larvae of *D. nasuta* was studied by employing the technique of DNA fibre autoradiography. The replicon properties in these two cell types was found to be remarkably similar.

Materials and Methods

Flies and larvae of a wild type strain (Varanasi) of *D. nasuta* were reared under standard laboratory conditions of food and temperature ($20° \pm 1°C$). Brain ganglia and wing imaginal disks were dissected out from late third instar larvae.

[3]H-thymidine labelling of brain ganglia and imaginal disks —'Step-down' protocol of ^{3}H-thymidine labelling[16] was used to analyse the replicon properties in the two cell types. Brain ganglia and imaginal disks from about 20 individuals were separately labelled at 24°C with the 'hot' ^{3}H-thymidine (act. 500 μCi/ml; sp. act. 46 Ci/mM, Amersham) pulse for 15, 30 or 60 min. Immediately thereafter, the tissues were washed with medium containing $10^{-4} M$ non-radioactive thymidine ('cold' thymidine) and incubated in the 'cold' thymidine medium at 24°C for 5 min. The tissues were then transferred to fresh medium at 24°C for the 'warm' pulse of ^{3}H-thymidine (act. 100 μCi/ml; sp.act. 9 Ci/mM, BARC, Trombay). The warm pulse was terminated by washing the tissues with ice-cold medium. The medium used for labelling or incubation was the modified Poels' medium[17].

Cell lysis and DNA fibre autoradiography — The labelled tissues were first lightly homogenized in a small glass homogenizer containing about 20 ganglia or 20 pairs of wing imaginal disks per 100 μl of chilled Poels' medium to dissociate individual cells. About 5 μl of the cell suspension was taken at one end of a subbed or albuminised slide alongwith an equal amount of the lysis solution (1% sodium-dodecyl sulphate (SDS) and 0.05 M Na-EDTA)[18]. The two solutions were allowed to mix for 1 min to effect cell lysis. The lysate was spread over the slide with the help of a thin cover glass. The slides were dried, treated with 5% trichloroacetic acid at 4°-6°C for 10 min, washed under running water and dehydrated in ethanol grades. Finally, they were coated with 1:1 diluted Ilford K5 nuclear emulsion, exposed in dark at 4°-6°C for about 6 months, developed in Kodak d19b, fixed in acid fixer, washed and dehydrated.

Scoring of the DNA fibre autoradiograms — The rate of fork movement (dense track sizes), replicon size (centre to centre distances) and other parameters were evaluated from photomicrographic prints of the labelled arrays following the usual considerations[19-21]. All those arrays suitable for analysis were photographed at an initial magnification of 64x. The final magnification of the prints was 384x. In certain cases of very short tracks in which the hot and warm tracks were not distinguishable at this magnification, the initial negative magnification was

128x and the final prints were 640x. All measurements were done using scale (with 0.5 mm division) and a divider to minimize the inaccuracy in direct measurements under the microscope[19].

Results

Types of labelled arrays in larval brain ganglia and imaginal disk preparations — The patterns of labelled arrays in the DNA fibre autoradiograms of larval brains and imaginal disks were, in general, very similar. Majority of the autoradiograms in both cell types showed typical long arrays of tandem 'hot' tracks, each flanked on either side by 'warm' tails (Fig.1). In the 60 min hot pulse samples of brain ganglia as well as imaginal disks, another class of labelled arrays was also frequently seen. These appeared as irregular groups of small arrays (Fig.2) each having a few dense tracks of relatively uniform and smaller size (less than 7 μm). In rare instances these smaller dense tracks were tandemly arranged to make a long array (Fig. 2a). The short dense tracks in these small arrays, particularly in imaginal disks, were often not flanked by warm tail on one or both sides (Fig. 2b). These two distinct classes of labelled arrays were designated as the types I and II, respectively. In the 60 min hot pulse samples, 27 arrays out of 134 arrays in brain ganglia and 35 out of 117 arrays in imaginal disk preparations were of type II. Several arrays in the 15 and 30 min samples of brain ganglia
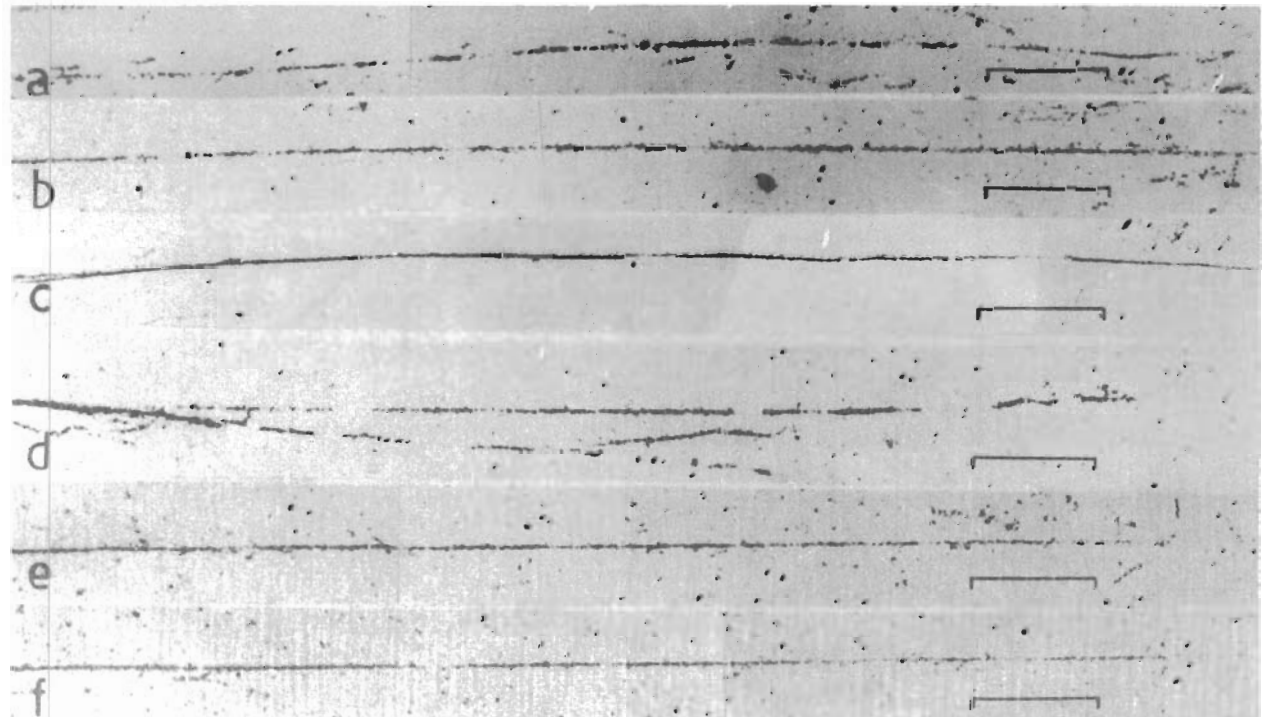

Fig. 1 —Type I arrays from brain ganglia (a,b,c) and imaginal disks (d,e,f) labelled with 'hot' ³H-thymidine pulse for 15 min (a,d), 30 min (b,e) or 60 min (c,f) followed in each case by a 'warm' ³H-thymidine pulse for 50 min. The bars in this and in Figs 2-4 represent 50 μm

and imaginal disks were seen which, in their general disposition resembled the type II arrays of 60 min samples, but whose calculated rate of fork migration was faster. Some examples of these are presented in **Fig. 3.** The examples in Fig. 3a-b are from brain ganglia labelled for 15 min. The labelled arrays in Fig. 3a contain several widely separated groups of active replicons with each group having one or more pairs of closely placed short dense tracks. In the example in Fig. 3b, a long array is formed by many closely placed active replicons. The examples in Fig. 3c-d are from 15 min pulse labelled imaginal disks. Fig. 3c shows a large number of irregularly disposed dense tracks, nearly all of them without flanking warm tracks. Several autoradiograms of this type were seen in 15 min pulse labelled imaginal disk preparations but fewer in brain ganglia. The labelled arrays in Fig. 3d from imaginal disks contain many closely placed tandem dense
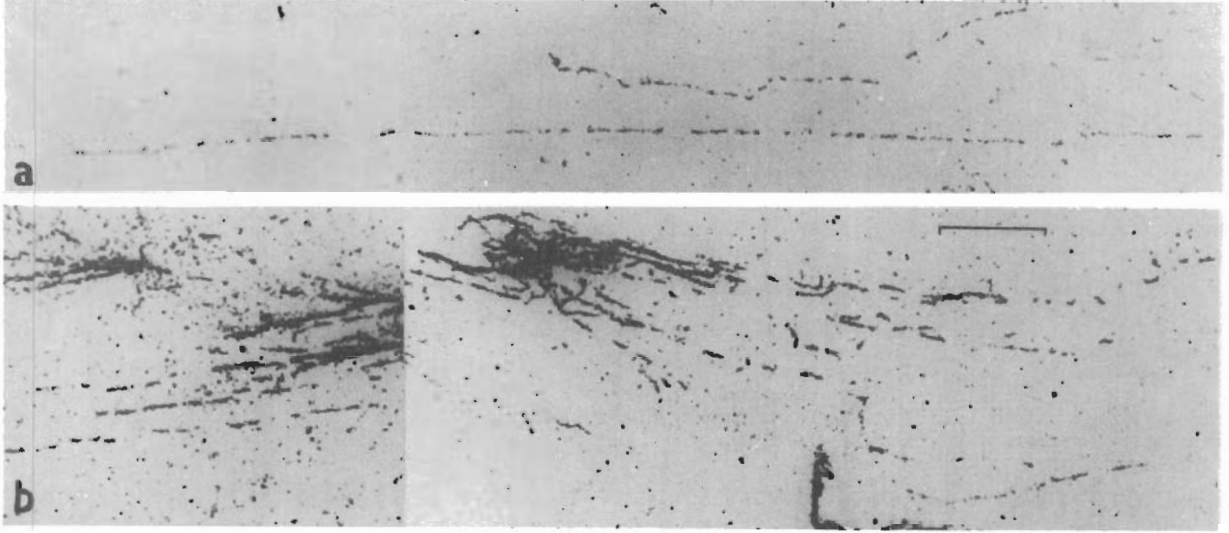

Fig. 2—Type II arrays from brain ganglia (a) and imaginal disks (b) labelled with 'hot' pulse of ^{3}H-thymidine for 60 min and 'warm' pulse for 50 min. in (a) only a part of the long array (about 788 μm) is shown. The active replicons in these arrays may (a) or may not (b) be flanked by warm tails
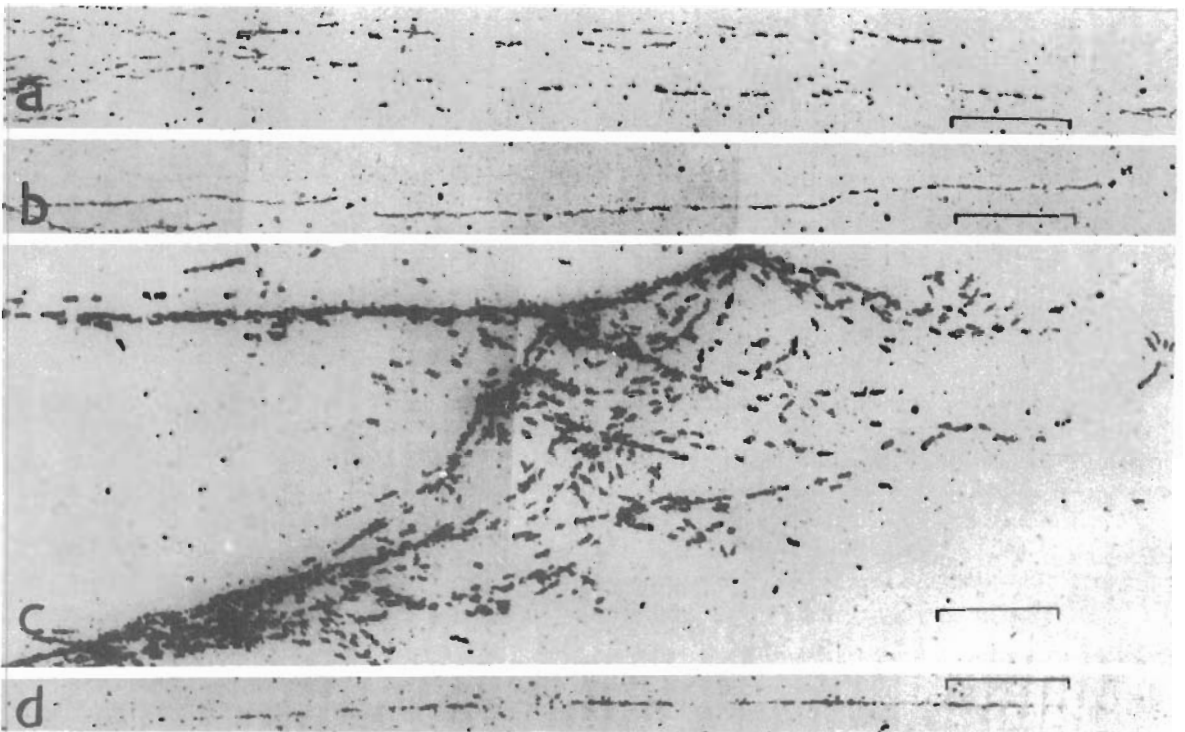

Fig. 3—Autoradiograms from 15 min 'hot' ^{3}H-thymidine pulse labelled samples of brain ganglia (a,b) and imaginal disks (c,d) showing certain unusual features as described in the text

tracks. Arrays of the types illustrated in Fig. 3 were classified as type I for the purpose of measurement of replicon size and rate of fork migration because their replicon sizes and fork migration rates overlapped with those of typical type I arrays like those in Fig.1.

Type I replicons

(a) Direction of movement: The 'step-down' protocol of hot and warm pulses permits differentiation between uni- or bidirectional migration of the replication fork on the basis of the absence or presence of flanking warm tails on one or both sides of the dense tracks[20]. The type I arrays in the 15 min samples of brain ganglia and imaginal disks were examined for the disposition of warm tails. The data presented in Table 1 show that about 33 to 35% of the dense tracks had warm tails on both sides. Out of the 622 dense tracks examined in 152 arrays in brain ganglia sample, 232 had a warm tail on only one side; about 60% of these showed a pre-pulse gap while the remaining were without any prepulse gap. In imaginal disks also, 247 tracks out of the total of 630 tracks analysed in 154 arrays, had a warm tail on only one side and among these about 42% were with a prepulse gap. Examples of arrays in which most of the dense tracks showed a warm tail on only one of the two forks are presented in Fig.4. About 25-29% of the analysed tracks did not show any warm tail (Table 1). Interestingly, a majority of the tracks without the flanking warm tails on either side were seen in arrays of the type shown in Fig.3.

(b) Replicon size: The size of a replicating unit (replicon) is empirically estimated by measuring the distance between adjacent origins (center to center or o—o distances)[16]. Since several of the dense labelled tracks were not flanked by warm tails (see above) it was of interest to know if they differed in some ways from those with flanking warm tails. Arrays without the warm tails do not permit an unambiguous identification of the origin point. However, as a best approximation, the origin point was identified as the

Table 1—Disposition of Labelled Hot Tracks with Flanking Warm Tails and Prepulse Gap between the Diverging Forks in 15 min 'Hot' + 50 min 'Warm' Pulse Labelled Autoradiograms of Brain Ganglia and Imaginal Disks

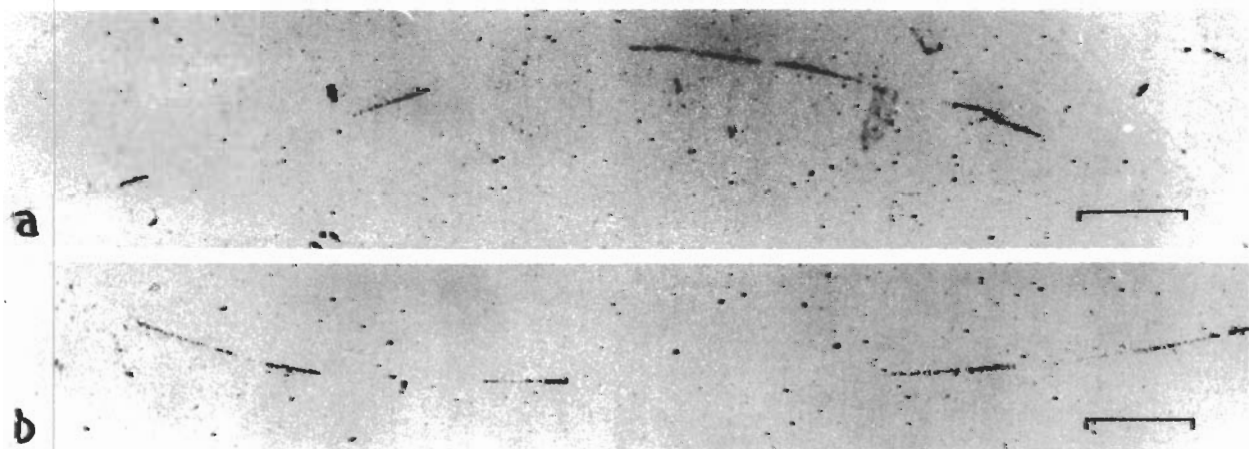| Type of 'hot' segments	Brain ganglia		Imaginal disks	
	No. (& %) of tracks	% tracks with prepulse gap	No. (& %) of tracks	% tracks with prepulse gap
Tracks with both forks flanked by warm tails	208 (33.4)	45	225 (35.7)	42
Tracks with single fork flanked by warm tail	232 (37.3)	60	247 (39.2)	42
Tracks without any flanking warm tail	182 (29.3)	—	158 (25.0)	—
Total No. of tracks analysed	622	—	630	—

Fig. 4 — Examples of type I arrays from 15 min 'hot' pulse sample of imaginal disks showing tandem tracks flanked by warm tails only on one side; several of the dense tracks do not show a prepulse gap
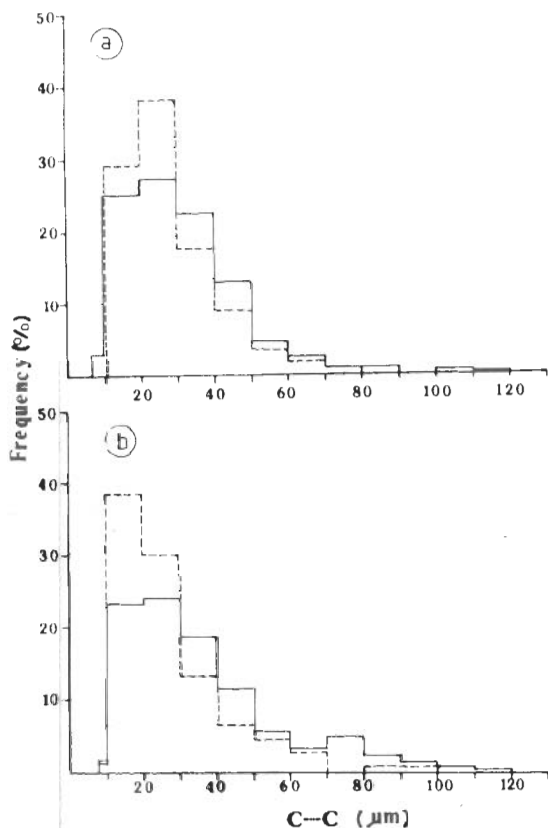
Fig. 5—Frequency distributions of o—o distances in 15 min pulse labelled type I arrays in which dense tracks are either flanked (solid lines) or are not flanked (broken lines) by warm tails from brain ganglia (a) and imaginal disks (b). The modal values of replicon sizes in arrays with and without warm tails in brain ganglia (22.9 and 23 μm, respectively) and in imaginal disks (21.3 and 18.1 μm, respectively) are similar

center point between two similar sized tracks, presuming that these two tracks reflected the two diverging forks[21]. A comparison of the distribution of o—o distances in 15 min samples of brain ganglia and imaginal disks between arrays with (N = 357 and 368, respectively) and without warm tails (N = 113 and 106, respectively) revealed (see Fig.5) that the replicon sizes between the two did not vary significantly. Therefore, in all samples (Fig.6), arrays with and without warm tails were considered together for estimating the replicon sizes. As expected, the o—o distances were larger in the longer pulse samples (Fig.6) due to fusion of advancing forks of neighbouring replicons. Therefore, the replicon sizes were estimated from the 15 min samples. The replicon size in 15 min samples ranged from 4.5 to 112 μm in brain ganglia and from 7.7 to 142 μm in imaginal disks; the modal values in these two cell types were also similar being 22.8 and 19.5 μm respectively (see Table 2). The mean o—o distances in these samples were 30.2 ± 0.8 μm for brain and 34.8 ± 1.1 μm for imaginal disks. The modal value of the o—o distances in the 15 min pulse samples were taken to represent the average replicon size, since the mean may be an overestimate.

(c) Rate of fork migration: The rate was calculated by measuring the length of dense track formed during a given pulse time. Considering the replication to be bidirectional, only one fork or the average length of two forks was taken for track length measurement. The rate was estimated from only those forks which were associated with flanking warm tails as well as with a prepulse gap. The frequency histograms of track sizes

Table 2—Dense Track Sizes (Single Fork), Rates of Fork Migration and Replicon Sizes in Type I and Type II Arrays in Different Samples of DNA Fibre Autoradiograms of Brain Ganglia and Imaginal Disks of *D. nasuta* Larvae

[Figures in parantheses indicate sample size in each case]

Tissue	Array type	Hot pulse (min)	Mean dense track length (± SE, μm)	Av. rate of fork migration (μm/min)	Modal value of o—o distance (μm)	Number of arrays
Brain Ganglia	Type I	15	6.6 ± 0.2 (331)	0.44	22.8 (470)	152
		30	8.7 ± 0.3 (142)	0.29	30.0 (296)	84
		60	14.7 ± 0.9 (93)	0.24	36.5 (371)	107
	Type II	60	4.1 ± 0.1 (346)	0.07	18.3 (146)	27
Imaginal Disks	Type I	15	7.8 ± 0.3 (290)	0.52	19.5 (474)	154
		30	9.9 ± 0.6 (163)	0.33	18.9 & 40.0 (268)	84
		60	15.1 ± 0.7 (112)	0.25	42.4 (283)	82
	Type II	60	5.1 ± 0.3 (248)	0.08	18.5 (89)	35

(single fork) in the different pulse samples of the two tissues are presented in Fig. 7. The mean sizes of the hot tracks in brain ganglia in 15,30 and 60 min samples were 6.6 μm (range: 2.5 to 20.8 μm), 8.7 μm (range: 1.5 to 21.8 μm) and 14.7 μm (range: 3.25 to 66.0 μm),

respectively (Table 2). The mean sizes of the labelled hot tracks in imaginal disks in the 15, 30 and 60 min samples was 7.8 μm (range: 1.3 to 27.5 μm), 9.9 μm (range: 2.8 to 36.2 μm) and 15.1 μM (range: 2.6 to 42.9 μm), respectively (Table 2). For brain ganglia, the mean rates of fork migration in 15, 30 and 60 min pulse samples were 0.44, 0.29, and 0.24 μm/min, respectively; for the imaginal disks, these values were 0.52, 0.33, and 0.25 μm/min respectively (see Table 2). The mean rates for the 15 min samples were taken as the rates of fork migration in the type I arrays of the two tissues, respectively.

(d) Intra-array variation in the o—o distances and the rates of fork migration: As noted above, the replicon sizes and the rates of fork migration in the type I arrays were found to vary over a wide range in any given sample (Figs 5-7). To analyze if this variation was due to intra- or inter-array differences, the maximum and minimum o—o distances and the dense track sizes in each type I array of the 15 min samples of brain ganglia and imaginal disks were compared. Only those arrays were included in this analysis which contained at least four tandem active replicons.

The intra-array variability in the o—o distances was assessed by taking the ratio of the longest and shortest o—o distances in a given array. Frequency histograms of these ratios for brain ganglia and imaginal disk samples are presented in Fig. 8a. It is seen that in about 70% of the analysed arrays, both in brain ganglia and imaginal disks, the longest replicon was less than 2.5x longer than the shortest replicon in the same array. Generally the arrays containing longer replicons tended to have a greater intra-array
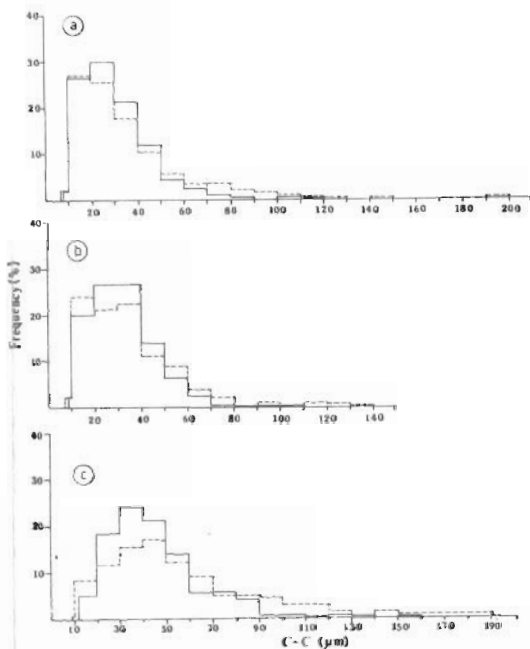

Fig. 6—Frequency histograms of o—o distances (C-C) on DNA fibres in type I arrays in brain ganglia (solid lines) and wing imaginal disks (broken lines) after a 'hot' pulse of ^{3}H-thymidine for 15 min (a), 30 min (b) or 60 min (c)
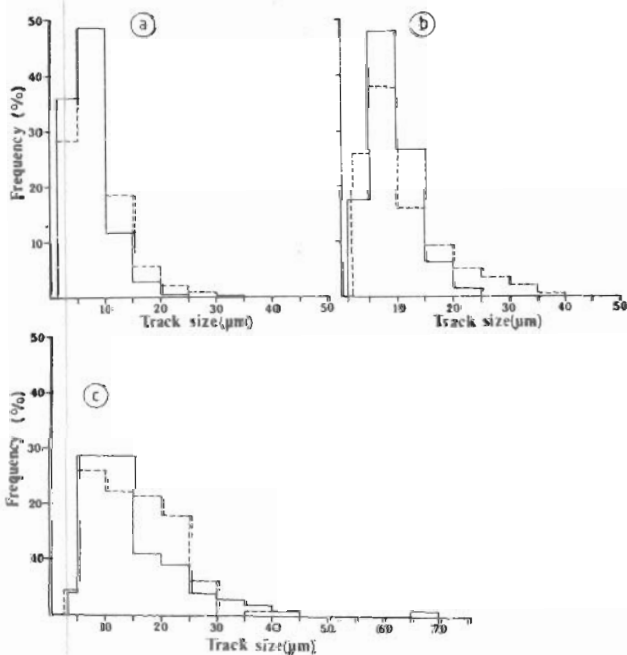

Fig. 7—Frequency distributions of labelled dense track lengths (single fork) in type I arrays from brain ganglia (solid lines) and imaginal disks (broken lines) after a 'hot' pulse for 15 min (a), 30 min (b) or 60 min (c)
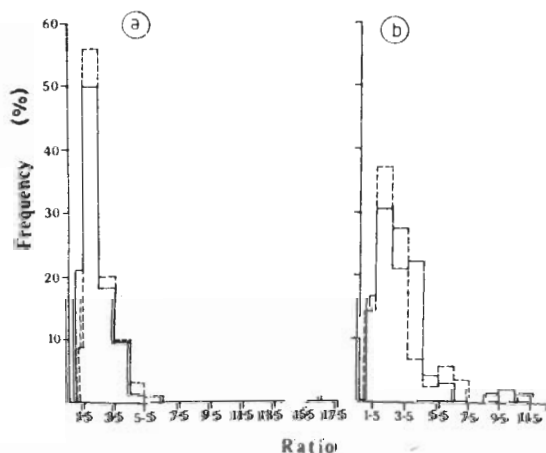

Fig. 8—Frequency distributions showing intra-array variations (ratio of maximum to minimum lengths in a given array) in the o—o distances (a) and dense labelled track sizes (single fork, b) in brain ganglia (solid lines, N = 72 arrays) and imaginal disks (broken lines, N = 91 arrays) labelled for 15 min. For further details, see text

variation, presumably due to fusion of adjacent replicons during the pulse period.

The intra-array variation in the rate of fork migration was also estimated by taking the ratio of the longest to shortest dense tracks in an array. The frequency histograms in Fig. 8b show that in about 50% of the arrays in both tissues, the fastest fork was less than 2.5x faster than the slowest fork in the array.

Type II replicons

In the type II arrays seen in 60 min samples, nearly all the dense tracks (single fork) were less than 7 μm except rare tracks which were longer. The frequency histograms of 0—0 distances and the dense track sizes in the type II arrays from the two tissues are presented in Fig.9. The modal replicon sizes in these arrays from brain ganglia and imaginal disks were 18.3

and 18.5 μm, respectively (Table 2), while the mean values were 23.1 ± 1.0 and 24.8 ± 1.2 μm, respectively. The average rates of fork migration in type II arrays in brain ganglia and imaginal disks were 0.07 and 0.08 μm/min, respectively (Table 2). All dense track, irrespective of the presence or absence of a prepulse gap or the flanking warm tails were included in this analysis.

Nearly 50% Of the type II arrays (17 out of 35 arrays analysed) in the 60 min pulse sample of imaginal disks did not have warm tails flanking the short dense tracks. The corresponding sample in brain ganglia showed much lower frequency of arrays lacking warm tails since only in 3 out of the 27 arrays, the dense tracks were not flanked by warm tails.

Discussion

Very few studies are available on the organization of active replicons in different cell type of *Drosophila*. While Blumenthal et al.[22] and Ananiev et al.[23] studied the active replicons in early embryos or in embryonic cells of *D. melanogaster* in culture, those in brain ganglia and salivary glands of *D. virilis* larvae have been studied by Steinemann[4,24] and in salivary glands of *D. nasuta* larvae by Lakhotia and Sinha[25]. No previous information is available regarding the organization of active replicons in imaginal disks of *Drosophila* larvae.

Two distinct categories of active replicons were identified in the DNA fibre autoradiograms of wing imaginal disks as well as brain ganglia of *D. nasuta* larvae. These two types differed from each other primarily in their rates of fork migration (0.07-0.08 μm/min and 0.44-0.52 μm/min, respectively) and also in the overall disposition of arrays of labelled tracks (Figs 1-3). In the brain ganglia of *D. virilis* also two kinds of labelled arrays were noted[4]. The type II arrays analysed in this study appear to correspond to the shorter arrays reported earlier[4] in *D. virilis* brain cells since (i) both types have a slow rate of fork migration and shorter o—o distances, (ii) both display irregular groups of arrays consisting of relatively similar sized dense tracks and (iii) both are characterized by a more frequent absence of flanking warm tails.

The type II arrays seen in the present study also compare well in their rate of fork migration, o—o distances and the general disposition of labelled tracks in the arrays with the type II arrays reported in salivary gland polytene nuclei of *D. nasuta*[25]. Steinemann[4] correlated the short and irregular arrays in brain ganglia of *D. virilis* to replicating heterochromatin while Lakhotia and Sinha[25] suggested the type II replicons in salivary gland polytene nuclei of *D. nasuta* to be associated with late
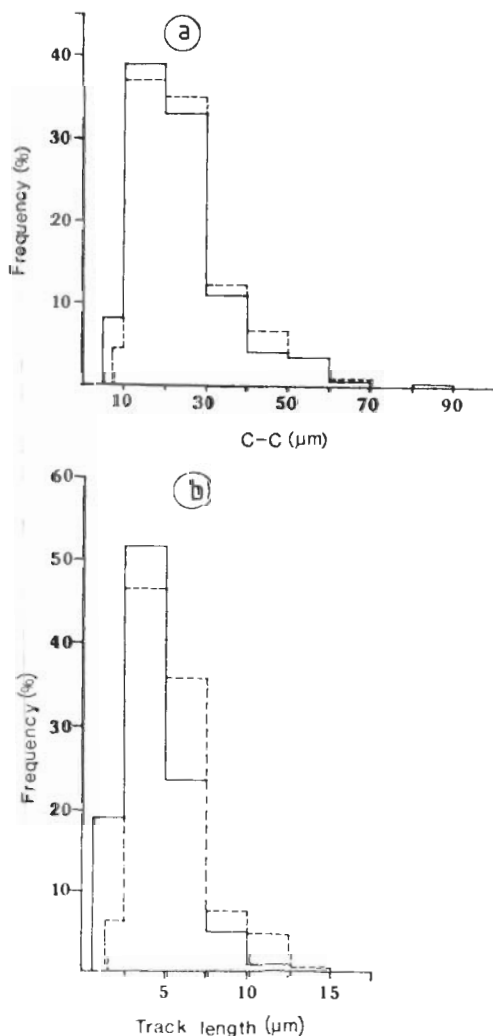

Fig. 9 Frequency histograms showing distributions of o—o distances (C—C, a) and dense track sizes (single fork, b) in type II arrays from 60 min "hot" pulse labelled brain ganglia (solid lines) and imaginal disks (broken lines)

replicating regions which also include chromocentric and intercalary heterochromatin. In view of these, it may appear that the type II arrays seen in brain ganglia and imaginal disks of *D. nasuta* larvae are also associated with heterochromatin replication although there is no direct evidence for this correlation in the present study (also see later).

The type I arrays seen in the present study are different in some respects from those in salivary glands of *D. nasuta* larvae[25]: the rate of fork migration in the type I arrays in brain ganglia and imaginal disks is nearly half that in polytene nuclei; likewise the replicon size in the type I arrays from brain ganglia and imaginal disks is nearly three times smaller than in salivary glands. These observations further show that the location of active origins and the rate of fork migration are subject to regulation in a tissue specific manner[16,22,26].

The organization of active replicons is expected to reflect the nuclear replication and cell cycle patterns in the tissue. As mentioned earlier, the brain cells may endoreplicate and/or divide mitotically. Among the mitotically dividing neuroblast cells, at least some complte one cell cycle within 1 hr while others apparently take longer[10,11,27]. At this point, we do not know if the two distinct categories of replicons seen in this study are related to (i) the progression of endoreplication and mitotic replication cycles, (ii) replication in cells with brief or more prolonged mitotic S-period or (iii) to the replication of eu- and heterochromatin regions. The observed intra-array variations in the different parameters of active replicons are compatible with any or all of the above possibilities.

The similarity in the organization of active replicons in brain gnaglia and imaginal disks of *Drosophila* larvae revealed in this study is remarkable. This may imply that the nuclear replication cycles in the two tissues may also be comparable[11]. The only difference between the replicon organization in brain ganglia and imaginal disks of *D. nasuta* larvae noted in this study

was the relatively higher frequency of smaller sized dense tracks without flanking warm tails in imaginal disks. The significance of such arrays with fixed termini remains to be known.

References

1 Gall G, Cohen E H & Polan M L, *Chromosoma*, 33 (1971) 319.

2 Friström J W, in *Biology of imaginal disks*, edited by H Ursprung and R Nöthiger (Springer-Verlag, Heidelberg, New York) 1972, 109.

3 Steinemann M, *Chromosoma*, 78 (1980) 211.

4 Steinemann M, *Chromosoma*, 82 (1981) 267.

5 Madhavan M M & Schneiderman H A, *Wilhelm Roux' Arch Dev Biol*, 183 (1977) 269.

6 Graves B J & Schubiger G, *Dev Biol*, 93 (1982) 104.

7 Garcia-Bellido A & Merrian J P, *Dev Biol*, 24 (1971) 61.

8 Pimpinelli S, Pignone D, Gatti M & Olivieri G, *Mutat Res*, 35 (1976) 101.

9 Tsuji H & Tobari J, *Exp Cell Res*, 125 (1980) 79.

10 Roy J K, Ph D thesis, Banaras Hindu University, Varanasi, 1983.

11 Lakhotia S C, *Proc DAE Symp on Cellular Control Mechanism*, (BARC, Trombay) 1982, 289.

12 Berendes H D & Keyl H G, *Genetics*, 57 (1967) 1.

13 Lakhotia S C & Kumar M, *Indian J Exp Biol*, 18 (1980) 1066.

14 Lakhotia S C, *Genetica*, 54 (1981) 247.

15 Endow S A & Gall J G, *Chromosoma*, 50 (1975) 175.

16 Callan H G, *Proc Roy Soc Lond*, B181 (1972) 19.

17 Sinha P & Lakhotia S C, *Indian J Exp Biol*, 18 (1980) 1059.

18 Lark K G, Consigli R & Toliver A, *J Mol Biol*, 58 (1971) 873.

19 Probst H, Blütters R & Fielitz J, *Exp Cell Res*, 130 (1980) 1.

20 Hand R, *J Cell Biol*, 64 (1975) 89.

21 Van't Hof J & Bjerknes C A, *Chromosoma*, 64 (1977) 287.

22 Blumenthal A B, Kriegstein H J & Hogness D S, *Cold Spring Harb Symp Quant Biol*, 38 (1973) 205.

23 Ananiev E V, Polukarova L G & Yurov Yu B, *Chromosoma*, 59 (1977) 259.

24 Steinemann M, *Chromosoma*, 82 (1981) 289.

25 Lakhotia S C & Sinha P, *Chromosoma*, 88 (1983) 265.

26 Taylor H J, in *DNA synthesis: Present & future*, edited by I Molineux and K Masamichi (Plenum Publication Corp, New York, London) 1978, 143.

27 Lakhotia S C, Roy J K & Kumar M, *Chromosoma*, 72 (1979) 259.