Replication in *Drosophila* Chromosomes: Part IV—Patterns of Chromosomal Replication in Salivary Gland Polytenic Nuclei of *Drosophila nasuta*

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In a previous report, it was shown that the nuclei of larval salivary glands of *D. nasuta* have a characteristic pattern of chromosome replication. This pattern was found to be consistent in all the nuclei examined, regardless of the stage of development of the larval salivary gland. The nuclei of these glands were found to be divided into two main regions: a core region and a peripheral region. The core region consists of chromosomes that have replicated, while the peripheral region consists of chromosomes that are in the process of replicating.

The replication pattern observed in *D. nasuta* is similar to that observed in other insects. The replication pattern is thought to be determined by the genetic information encoded in the DNA of the chromosomes. The pattern of replication is thought to be important for the proper segregation of chromosomes during cell division.

1. **Polytenic nuclei of larval salivary glands of *Drosophila* undergo a definite sequence of cyclic replication in which a synthetic phase (polytenic S-period) alternates with an interphase phase.** However, the absence of cell cycle stage dependent differences in morphology of polytenic nuclei has hindered the analysis of the temporal sequence of replication of different chromosome regions and the determination of the duration of a polytenic S-period. Nevertheless several indirect considerations have led to the general belief that the generally observed 3 categories of autoradiographic labelling patterns of *S*-thymidine pulse labelled polytenic chromosome of *Drosophila* viz., interband (IB) labelling patterns (with predominating labelling of dense band regions), occur in that order in a polytenic S-period in late third instar larval salivary glands. The initial stage of the polytenic S-period, represented by the interband (IB) labelling patterns (also termed "disperse discontinuities" or "IB patterns" by Chatterjee and Mukherjee), has been found to be rather brief in the *Drosophila* species analyzed in detail.

2. **Materials and Methods**

A wild strain of *D. nasuta* (Vajram) has been used for this study. These larvae were reared on standard agar-cornmeal-broth-sugar yeast food at 24 ± 1 °C.

To obtain healthy and synchronized larvae, eggs were collected in food filled petri dishes for 26-66 min. In the larva of *D. nasuta* the volume of the anterior and posterior spiracles changes during third instar and this colour change was taken as the external marker to obtain a more synchronously growing population of *D. nasuta*./*n/a/
larvae. About 30-34 hr prior to anterior spiracle eversion, the posterior pair of spiracles develop a black pigment stripe at their base and this stage is recognized as 8 hr black spiracle stage. The stage of anterior spiracle eversion is referred to as 0 hr prepupa. Salivary glands from late third instar larvae (between 0 hr and 24 hr black spiracle stage) and from 0 hr to 4 hr old prepupa were dissected out in Drosophila Ringer (pH 7.2). The first group of larvae was dissected between 1 and 8 hr, the second group between 10 and 10 hr and the third group between 18 and 24 hr after the black spiracle stage. In each case the excised salivary glands were labelled for 10 min with H- thymidine in Ringer (250 &muCi/ml; sp. act. 10.4 Ci/mM; B&RC, Troms&oslash;). Squash preparations of labelled glands were processed for autoradiography as described earlier. To facilitate analysis of the autoradiograms and to serve as a standard reference, a photomap (Fig. 1) of the polytene chromosomes of D. nasuta has also been prepared from phase-contrast photomicrographs of aero-oeno-carmine stained temporary squash preparations of late third instar larval salivary glands.

Results
Polytene chromosomes of D. nasuta.
The metaphase karyotype of D. nasuta consists of acrocentric X, metacentric 2d, acrocentric 3d and dot like 4th chromosomes. Correspondingly, salivary gland polytene chromosome complement of D. nasuta consists of 4 long arms and a short arm. The 4 long arms correspond, respectively, to X, 2L, 2R and 3d chromosomes while the short arm represents the 4th chromosomes. The chromosome centre is very small made up largely of a compact rounded alpha heterochromatic mass with little beta heterochromatic regions at the bases of different arms. Among the long arms of D. nasuta polytene nuclei, 2L is the shortest while chromosome 3 is the longest. The X is slightly longer than 2L, but is shorter than 2R. In photomap (Fig. 1) of D. nasuta polytene chromosomes the X, 2L and 2R have been divided into 20 divisions each while the long third chromosome is divided into 40 divisions. X-chromosome has divisions 1 to 20 from the free tip to centromeric end; 2L has divisions 21-40 from tip to centromeric end; the divisions (41-60) on 2R are numbered from centromeric end to the free tip while on chromosome 3, the divisions are numbered from 61-100 from tip to basal region. Chromosome 4 is very small and only one division is recognised on this arm. Each division is further sub-divided into 3 sections (A, B and C) with each section starting from an interband (Fig. 1). Each section is further divisible into several bands (details not given here). It may also be noted that many larvae of the strain used in this study carry an inversion on 2L between 2SC and 38B.

Patterns of H-thymidine incorporation in polytene chromosomes.

General patterns of autoradiographic labelling and their frequencies in larval and prepupal glands.- The general patterns of autoradiographic labelling of different polytene nuclei in D. nasuta following a pulse of H-thymidine are essentially similar to those described earlier in D. kikkawai, and as in that species, we have classified the H-thymidine labelled polytene nuclei of D. nasuta also into the following categories: (i) low interband (LIB), (ii) mid interband (MIB), (iii) heavy interband (HIB); (iv) medium continuous (2C); (v) heavy continuous (3C); (vi) heavy discontinuous (3D); (vii) medium discontinuous (2D); and (viii) late or low discontinuous (1D) type of labelling patterns. We presume that (see discussion) as in D. kikkawai, in late third instar larval polytene nuclei of D. nasuta also the labelling patterns (i) to (vii) occur in that sequence during the progression of a polytene S-period from initiation to termination; in other words, the LIB type of nuclei are presumed to be in the very early and 1D type nuclei in the late phase of a given polytene S-period. Representative examples of these labelling patterns are illustrated in Fig. 2a-g to show the general distribution of autoradiographic labelling in these categories. As can be seen from the examples in Fig. 2a-g, the LIB nuclei have a low labelling of only one or two puffs and none or a few interbands while in MIB and HIB types, progressively more puffs and interbands and light bands appear labelled. In different HIB type nuclei the labelling of the interbands and the puffs varies from moderate to heavy (Figs. 2c, 4c and 4d), but most of the dark bands remain unlabelled. In 3D to 1D patterns, the interband and puff regions become progressively unlabelled and also the intensity of labelling and the number of labelled bands decreases progressively as originally described by Rodman. The chromosome centre in polytene nuclei of D. nasuta is largely made up of alpha heterochromatin. In all the nuclei labelled with H-thymidine, irrespective of their labelling patterns, the alpha heterochromatic mass was always seen to be distinctly unlabelled (Fig. 3a-g).

Preparations from larvae (male and female) and prepuca of different age groups (see material and methods) were scored for the frequencies of different labelling patterns. As can be seen from the data in Tables 1 and 2, the frequencies of the different labelling patterns vary in a characteristic manner with larval and prepupal age. It is seen that in both male and female larvae the frequency of unlabelled nuclei increases with increasing larval age. However, in 10-16 hr black spiracle stage larvae, the frequency of
Fig 2: Autoradiographs of 3H-thymidine probe labelled polytene chromosomes of last third instar larvae of *D. melanogaster* to show the general territories of the 8 categories of chromosomal labelling patterns classified in this species. Some of the puff sites on 2R and X, seen in the different montages, are indicated. × 1200. a. Montage of a complete male nucleus showing low (highband) L1R1 type labelling with only puff 44A on 2R being labelled. All other regions including the X-chromosome are completely unlabeled. The intranuclear DNA is also labelled (for details of intranuclear labelling, see ref. 19.8). Part of a mid interband MBB type labelled maverick. Note the labelling of several interbands and puffs in addition to 44A puff.
Fig. 2. a. Part of a heavy interband (HB) type labelled nucleus showing a heavy labelling of most puffs and interbands. Many bands are distinctly unlabelled (arrows). b. Part of a heavy continuous (C) type labelled nucleus. c. Part of a heavy discontinuous (DD) type labelled nucleus with some puff and interband sites being unlabelled. d. Parts of medium (MD) and low (LD) discontinuously labelled nuclei, respectively. Note the progressively reduced labelling of band regions. While most puffs and interbands are unlabelled in these patterns, the 48A puff is well labelled.
unlabelled nuclei in female larvae is much higher (46.0%) than in male larvae (17.9%) while in other 2 age groups the frequency of unlabelled nuclei is nearly similar in male and female larvae. Among the labelled nuclei it is seen that the frequencies of interband patterns (particularly L1B and M1B types) increase with larval age while the continuous (3C) and discontinuous (2D and 1D) patterns show a decline. The very frequent frequency of L1B and M1B patterns in larvae of either sexes (particularly in the 18-24 hr black spiracle stage) is very interesting. Analysis of frequencies of different labelling patterns during the first 4 hr after anterior spiracle excision shows that in these salivary glands, the labelled nuclei are fewer, and among these, the interband patterns are totally absent (Table 2). While in 0 hr prepupal glands, the 3C type patterns are seen in slightly more than 25% of labelled nuclei, in the later stages, these patterns are no more present. Likewise, the 3D and 2D patterns also become fewer in older prepupae, and in 4 hr prepupa only 2D (11.90%) and 3D (8.90%) types of nuclei are seen (Table 2).

1H-thymidine labelling of the 48 h puff—The most important feature of the polytene chromosomes of D. melanogaster is the pattern of 1H-thymidine incorporation in the puff 48A on chromosome 3R. It is known to incorporate 1H-thymidine in all labelled nuclei and in many of the L1B type of nuclei, this is the only site in the nucleus (except the nucleolus), which is labelled (Fig. 2a). In most of the 1D type nuclei also this puff shows a significant 1H-

Fig. 3a–g—Parts of autoradiographs of polytene nuclei of D. melanogaster show site of incorporation of 1H-thymidine in alpha heterochromatic (arrow) in L1B (a), M1B (b), H1B (c), 2C-3C (d), 3D (e), 2D (f) and 1D (g). Type labelled nuclei: × 1200

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thymidine incorporation (Fig. 2a). To obtain a more detailed information on the replicative organization of this puff, we have counted the number of silver grains on 48A and 4 other (4C, 46C, 44A and 43C) puffs on 7R in nuclei showing different types of H-thymidine labelling patterns in preparations of larval salivary glands (Table 3). In this analysis the data of HIB, 2C and 3C types of nuclei could not be obtained because of very low number of scorable HIB and 2C nuclei while in 3C type nuclei, a very heavy labelling of all chromosome regions does not permit a precise grain count on the puff regions. Likewise for the 3D type nuclei, only very few nuclei could be scored since in others the grain density was too high. Nevertheless the

(Fig. 4 - Examples of interband type labelled polytene chromosomes of male D. melanogaster to show differential replication of X-chromosome, a (20×), Autosome X-LIB type with a few puffs low labelled; the lightly stained male X is nearly unlabelled, except for 2-3 grains on a few sites like 3C puff, b) Autosome X-LIB type with several puffs and interbands labelled; the male X shows a lesser degree of labelling
data in Table 3 and other observations. (Fig. 2a-g) very clearly reveal interesting differences in the replicative organization of 48A and other puffs. All the 3 puff sites show maximum grain density in 3D (and in 3C) patterns. But only the 48A puff shows a pronounced labelling (with 10 or more silver grains on average) in 1D as well as 3D type nuclei. The other puff sites are mostly unlabeled or very low labelled in these two types of labelled nuclei, particularly in 1D type where the very low labelling in these puffs is seen in only a few nuclei and that too nearer the adjacent band region.

On the other hand, in 48A puff, the silver grains appear more generally distributed over the puff area (see Fig. 2a and g), indicating that the 48A puff DNA is replicating even in the late S (see Discussion). Another significant difference between the 48A and other puffs is the several times higher mean grain density on 48A puff compared to the other sites in all types of labelled nuclei (Table 3).

Labelling patterns of X-chromosome in male and female polytene nuclei of D. hyaza — As in other species of Drosophila[19], H-thymidine incorporation

Fig. 4 - 9: Autoradiography with labelling intermediate between M1H and H1H types. The male X shows nearly similar labelling intensity as autosome 8. Autosomes with typical H1H type labelling; the male X shows a much heavier labelling, approaching the 3C level
Table 3 - Mean Grain Number on Five Puff Sites on Arm 2R with Respect to Different "{\textit{H}}"-thyminde Labelling Patterns

<table>
<thead>
<tr>
<th>Labelling pattern</th>
<th>4\textit{AC}</th>
<th>4\textit{BC}</th>
<th>4\textit{DC}</th>
<th>4\textit{EC}</th>
<th>4\textit{LC}</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHB</td>
<td>9.25 ± 0.57</td>
<td>2.45 ± 0.14</td>
<td>1.21 ± 0.17</td>
<td>0.94 ± 0.11</td>
<td>2.15 ± 0.19</td>
</tr>
<tr>
<td>DB</td>
<td>10 ± 1</td>
<td>2.71 ± 0.33</td>
<td>2.95 ± 0.19</td>
<td>2.64 ± 0.19</td>
<td>3.72 ± 0.39</td>
</tr>
<tr>
<td>1D</td>
<td>5 (5)</td>
<td>6 (6)</td>
<td>9 (9)</td>
<td>5 (5)</td>
<td>10.00</td>
</tr>
<tr>
<td>2D</td>
<td>58 ± 0.77</td>
<td>2.72 ± 0.61</td>
<td>2.66 ± 0.31</td>
<td>4.13 ± 0.90</td>
<td>4.87 ± 1.13</td>
</tr>
<tr>
<td>3D</td>
<td>10 (10)</td>
<td>1.86 ± 0.25</td>
<td>1.84 ± 0.21</td>
<td>1.62 ± 0.51</td>
<td>2.38 ± 1.42</td>
</tr>
</tbody>
</table>

Discussion

The present autoradiographic studies on "{\textit{H}}"-thymidine pulse labelled polytene nuclei of {\textit{D}}. \textit{melanogaster} reveal several interesting features of polytene replication in this species. The high frequency and the variety of interband type of "{\textit{H}}"-thyminde labelling patterns in late third instar larval polytene nuclei of \textit{D}. \textit{melanogaster} are striking. A comparable replicative organization of polytene chromosomes has been described only in larval salivary glands of \textit{D}. \textit{kikowaei}4. As described in this paper for \textit{D}. \textit{kikowaei} (also see ref. 3, 4 and 11 for other species of \textit{Drassophila}), it appears that in late third instar larval polytene cells of \textit{D}. \textit{melanogaster}, a given polytene S-period starts with initiation of replication at a few specific puff sites, with other puff and interbands becoming active sequentially during M1B and 1B phases and finally followed by replication of different bands. In contrast the nuclei in which only the puff 4A is labelled are very interesting. We have reported earlier4 that in some "{\textit{H}}"-thymidine pulse labelled polytene nuclei of \textit{D}. \textit{kikowaei} also only the puff site 11E on arm E appears labelled and we had suggested that the single puff labelled nuclei represent the very initial stage of a polytene S-period. The same may be suggested for the specific labelling of 4A puff in some 1B type polytene nuclei of \textit{D}. \textit{melanogaster} observed in this study. It is significant that in all polytene nuclei we did not see any single puff labelled or even more advanced 1B (M1B or H1B) type labelled nuclei. This further confirms our belief that the 1B pattern represents the initial stages of polytene S-period since it has been shown by Rodin16,17 (also see ref. 1) that in prepared salivary glands, new initiations of polytene S-period do not occur. The 2D and 1D type patterns, which become progressively more common among labelled nuclei with increasing prepupal age, obviously represent the later stages of a polytene S-period.

The data on the frequencies of different "{\textit{H}}"-thymidine labelling patterns in polytene nuclei from different age-groups of larvae show that the LHB type and to a lesser extent the M1B type labelling patterns are very commonly seen, particularly in 1B-24 for black spicular age-group. Since, as discussed above, the 1B type pattern represents the initial stage, this would imply that there is a synchronous initiation of a new replication cycle in many nuclei of salivary glands at this stage. This apparent synchrony of initiation of a new replication cycle in groups of polytene nuclei in \textit{D}. \textit{melanogaster} permits us to analyse if the synchronously initiated nuclei with varying polytene lengths proceed synchronously through the S or they have a varying duration of the S-period and a corresponding variation in the duration of different phases of an S-period, as has been suggested earlier4. This aspect is now being analysed in our laboratory.

The replicative organization of 4A puff in \textit{D}. \textit{melanogaster} is interesting. This puff appears to incorporate "{\textit{H}}"-thymidine throughout a polytene S-period as evidenced by its labelled appearance in all types of labelled nuclei from LHB to 1D. Except in \textit{D}. \textit{kikowaei}4, in most other species of \textit{Drassophila}4,16,17, the early replicating puff regions have been seen to complete their replication cycle before the 2D or 1D stages. The replicative organization of the 4A puff in \textit{D}. \textit{melanogaster} is obviously different. In view of the several times higher grain density at 48A (in comparison to other puff sites, Table 3) in all labelled nuclei, and its extended period of "{\textit{H}}"-thymidine incorporation, it may be envisaged that the 4A puff is involved in "extra" DNA synthesis. However this possibility may...
be negated by two observations. Firstly the pattern of quantitative variation in a 94-thymidine incorporation during the progression of a polytene S-period at 48A site is similar to that of other sites, being low in early S (LIB and MIB type nuclei), very high in mid-S (LIB-2C and 3C-3D type nuclei) and again declining in late-S (2D1 and 3D-1 type nuclei) phase. Secondly it has also been observed that the 48A pupal size revalidates the same irrespective of low or high degree of thymidine incorporation in this focus in different labelling patterns. Nevertheless, further analysis of the molecular and cytochemical organization of replicating sequences at 48A pull of D. anata is needed to understand the significance of the unusual replicative pattern of this pupal site.

The lack of thymidine incorporation in the alpha heterochromatin in polytene nuclei of D. anata is as expected since from the other studies in different species it has been observed that the alpha heterochromatin does not polytize in polytene nuclei of Drosophila. Furthermore, using fluorescence techniques, it has been shown earlier that in polytene nuclei of D. anata, the bulk of chromocentre region is made of non-replicative alpha heterochromatin. The large size and the distinction of the alpha heterochromatin in polytene nuclei of D. anata permits an easy analysis of its replicative organization by autoradiographic technique also.

The faster completion of replication of X-chromosome in polytene nuclei of different species in male Drosophila has been studied in detail in several earlier studies. Similar detailed studies of X-chromosome in male and female polytene nuclei during the early S period are fewer. Häggé and Kallichris compared the 94-thymidine labelling of X-chromosome in initial (on baso) labelling pattern in male and female polytene nuclei of D. melanogaster and suggested that "in the initial stage of replication the male X-chromosome is in a base replication phase than the X-chromosome in female or autosomes". However in another study, a heavier labelling of male X-chromosome in interband type of labelled nuclei of D. melanogaster has been stated to be almost absent. On the other hand Mukherjee and Chatterjee have reported a heavier labelling of male X in polytene nuclei of D. pseudoobscura showing HJ3 (late D1 pattern) of Mukherjee and Chatterjee and 2C type of autosomal labelling. In a study of replication in polytene nuclei of F, hybrid of D. anata and D. anubhavii, Merri has reported that the X-chromosome of male may undergo the replication earlier than the autosomes. In the context the present observations in D. anata are interesting particularly since in this species it appears that the initial phase of replication in polytene nuclei is more extended. Our observation suggests that in D. anata, the initiation of replication on X-autosome sites, both in male and female, occurs at some autosomal sites (48A, pupa of 28) have already been initiated to replicate. However, relative to female X, the initiation at specific sites in X-chromosomes of male polytene nuclei of D. anata seems to occur slightly later since more sites on autosomes of male nuclei are labelled when the labelling on the male X-chromosome is first (pupal 7C) detectable. In D. anata, the X-chromosome in male nuclei appears to be more advanced in replication only than the H1B stage onwards. It may be noted that the early pattern described in previous studies in D. melanogaster or in D. pseudoobscura appear to be comparable to the H1B pattern of D. anata. Apparently, a replicon stage comparable to H1B and MIB patterns of D. anata either do not exist in these two species or it is too rare to be recorded. Thus, unlike the condition reported by Merri in the hybrid larvae, in D. anata, the male X does not start replicating earlier than the autosomes. Rather, the observations indicate that the initiation of male X may, in fact, be slightly delayed compared to the female X. But after this brief initial delay, the male X-chromosome soon replicates faster in comparison to the autosomes or the X-chromosome in female.

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References