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Replication in *Drosophila* chromosomes

X. Two kinds of active replicons in salivary gland polytene nuclei and their relation to chromosomal replication patterns*

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Abstract. DNA fibre autoradiography of highly polytenized nuclei in salivary glands of *Drosophila nasuta* larvae reveals two distinct types of active replicons. Type I replicons are longer (mean size = 64 μm), have a very high rate of fork migration (average rate = 0.95 μm/min) and generally occur in large arrays often extending over several thousand μm. In contrast, the type II replicons are smaller (mean size = 20 μm), slow replicating (average rate = 0.07 μm/min) and occur in short arrays containing only a few closely spaced active replicons. Evidence is presented that type I replicons are active in the early S and type II in the late S. Observations on autoradiographic labelling of partially lysed polytene chromosomes provide evidence for a lack of temporal and spatial agreement in the activation of origin points in homologous regions of the lateral polytene strands; these observations also suggest local variations in levels of polyteny within a chromosome. On the basis of this and other available information on replication in polytene chromosomes the possible roles of the two replicon types in the generation of the different ^{3}H-thymidine labelling patterns of polytene chromosomes are discussed.

Introduction

Various autoradiographic patterns of ^{3}H-thymidine labelling in different cytological regions of polytene chromosomes of *Drosophila* have been described in relation to the presumptive early and late phases of S period (Rudkin 1972; Hägele 1973; Roy and Lakhotia 1981). During the very early phase (the interband or IB-labelling phase), ^{3}H-thymidine is incorporated in a variable number of disperse or less dense regions and labelling ranges from low to heavy in a few to nearly all puffs, interbands and thin bands (Roy and Lakhotia 1981; Mishra and Lakhotia 1982a). This phase is followed by a continuous labelling phase during which all sites in a nucleus are moderately (2C) or heavily (3C) labelled. The C-labelling phase is followed in the late S by discontinuous (D) patterns of labelling during which replication continues in decreasing numbers of dense bands and heterochromatic regions (Rodman 1968). It has been inferred that the IB- and C-labelling phases together occupy a smaller part of the S period in polytene cells than do the D-labelling phases (Arcos-Terán 1972; Steinemann 1981b; Achary et al. 1981).

These autoradiographic labelling patterns of polytene chromosomes have been interpreted in the light of the classical concept of a cable-like polytene chromosome structure (Beermann 1972; Rudkin 1972). Since many bands complete their replication at different times in the late S, individual bands are often considered to be units of replication (Mulder et al. 1968; Rudkin 1972). The interband regions are presumed to contain very little DNA (Beermann 1972) and, therefore, are believed not to form separate replicons (Mulder et al. 1968; Blumenthal et al. 1973). The IB-labelling patterns are presumed to be due to the presence of initiation sites of band DNA in the adjoining interband (Mulder et al. 1968; Achary et al. 1981). It is also believed that the origin points on all lateral copies of a given polytene replicating unit are activated synchronously and that by the time the nucleus reaches the C-labelling phase all potential origin points have been activated (Steinemann 1981b). The different D-labelling patterns are thus presumed to result from differences in the duration of replication of different band regions (Rudkin 1972; Steinemann 1981b). Results of a recent DNA fibre autoradiographic study of polytene nuclei (Steinemann 1981b), however, suggest that replicons in these chromosomes correspond, on average, to DNA content of three-four bands, rather than to one band.

A new dimension has been added to the structural and replicative organization of polytene chromosomes by the suggestion (Sorsa and Virrankoski-Castrodeza 1976; Roberts 1979; Cowell and Hartmann-Goldstein 1980; Laird 1980) that there are local variations in the levels of polyteny in different euchromatic regions of a polytene chromosome. These local variations must have their basis in the patterns of replication. However, the conventional interpretations of the various ^{3}H-thymidine labelling patterns, do not provide a conceptual framework for generation of local variations in polyteny. Two other aspects of replication in polytene chromosomes, the molecular basis of which is obscure, are (1) the C-labelling patterns of all chromosome regions with a pulse of ^{3}H-thymidine as brief as 1–2 min (Mishra and Lakhotia 1982a) and (2) the enormous variation in the rate of ^{3}H-thymidine incorporation at any given independently replicating chromosome site during early as well as late stages of polytene S (Kalisch and Hägele 1973; Roy and Lakhotia 1981).

Paradoxically, while their polytene organization makes

* We take pleasure in dedicating this paper to our inspiring teacher Prof. S.P. Ray Chaudhuri on his completing 75 years of fruitful life

these chromosomes attractive for cytological studies, it also imposes serious limitations on correlating chromosomal labelling with molecular events in each of the many constituent chromatids. To attempt this correlation, we have used DNA fibre autoradiography to characterize the replicons active in polytene nuclei of *D. nasuta* at different stages of their S period. To analyze the replicons active during the early S period (IB phase), replicating polytene nuclei were synchronized in vivo at the early S period by feeding the larvae on medium containing 5-fluorodeoxyuridine (FdU) (see Achary et al. 1981; Sinha and Lakhotia 1983). The replicon properties from such synchronized early S nuclei were compared with those from late S nuclei (D patterns) prevalent in normal unsynchronized glands. Polytene nuclei had two distinct classes of replicons, which differed greatly in size and rate of fork migration and which were active at different stages of the S period. In this respect our results differ from those of the only other (Steinemann 1981b) DNA fibre autoradiographic study of polytene chromosomes. On the basis of the present results and other information on polytene replication, the possible roles of the two replicon types in the generation of different chromosomal labelling patterns are discussed.

Material and methods

Unsynchronized and synchronized salivary glands. Flies and larvae of *D. nasuta* (Varanasi) were reared on standard food at 24° ± 1° C. For ^{3}H-thymidine labelling the salivary gands were removed from female mid-third instar larvae (115–120 h postoviposition) either grown on the normal food or fed for the last 24 h on medium containing FdU (200 µg/ml, Sigma) as described earlier (Sinha and Lakhotia 1983). Salivary glands from normally grown larvae are referred to as unsynchronized since their replicating nuclei are at different phases of the polytene S period (Rudkin 1972; Roy and Lakhotia 1981); and those from the FdU-fed larvae, as synchronized since 70%–90% of their replicating nuclei are at the early S period (Achary et al. 1981; Sinha and Lakhotia 1983).

^{3}H-thymidine labelling. For analyzing replicon properties, a step-down procedure of "hot" (act = 1,000 µCi/ml; sp. act. = 45 Ci/mM, Amersham) and "warm" (act. = 100 µCi/ml; sp. act. = 5 Ci/mM, BARC, Trombay) ^{3}H-thymidine pulse labelling was followed. Unsynchronized glands were sequentially labelled at 24° C with hot and warm pulses for 10 + 50, 20 + 50 or 60 + 50 min; the synchronized glands were labelled for 10 + 50, 20 + 20, 30 + 50 or 60 + 50 min. During the hot pulse, only the inorganic salt constituents of the salivary gland culture medium (Sinha and Lakhotia 1980) were present in the incubation medium; the warm pulse was applied in the complete medium. To rapidly terminate the hot pulse the glands were thoroughly washed in complete medium supplemented with 10^{-4} M "cold" thymidine.

Cell lysis and DNA fibre autoradiography. Immediately after the warm pulse, the proximal third of the paired salivary glands was removed to exclude the low polyteny and anlage nuclei. The distal two-thirds were transferred to one corner of a clean, gelatin-subbed slide in a drop of lysis solution [0.01 M EDTA (ethylenediaminetetraacetate), 1% SDS (sodium dodecyl sulfate), 0.02 M Tris-HCl, 1 mg/ml pronase, pH 7.2–7.4]. Pronase (type I, Sigma) was added to promote complete separation of the many DNA fibres constituting a polytene chromosome. After 1 min in the lysis solution, the glands were gently teased apart with stainless steel needles to separate individual cells and to facilitate their lysis. Lysis was continued for 10–15 min at 24° C. Finally, the lysate was gently drawn into a thin film over the slide with a coverslip held at an angle on the lysis drop. The slides were air-dried, treated with 5% TCA (trichloroacetic acid) for 10 min at 4°–6° C, washed in running water and dehydrated through ethanol grades. The dried slides were coated with Kodak NTB 3 or Ilford K5 nuclear emulsion and exposed in the dark at 4°–6° C for 3 months. The autoradiograms were developed with Kodak D19B developer at 10° C for 10 min, fixed in acid fixer, washed and examined.

Scoring of the DNA fibre autoradiograms. The origin-to-origin distances (O–O), high density track lengths and rates of fork migration were estimated from the autoradiograms of spread DNA fibres following the usual methods of analyses (Callan 1972; Yurov 1979). All suitable arrays of labelling (containing at least three tandemly aligned dense tracks of silver grains formed during the hot pulse) were photographed and printed at × 240 (type I arrays) or × 480 (type II arrays) magnification. The lengths of the dense tracks (calculated for single fork, see legend to Fig. 5) and the distances between adjacent origin points were measured from the photomicrographs with calipers and a ruler.

Results

Salivary gland from normally grown (unsynchronized) or FdU-fed (synchronized) mid-third instar larvae were sequentially labelled with hot and warm pulses of ^{3}H-thymidine (Callan 1972) and lysed to spread the labelled DNA fibres. In preliminary studies, the labelled salivary glands were lysed in the usual SDS-EDTA lysis medium. In these preparations, distinct autoradiograms of well-spread long DNA fibres were rare since in most cases the polytene bundles of DNA fibres tended to remain together. Therefore, in the present study, the lysis conditions were modified by adding pronase to the SDS-EDTA lysis medium and extending the lysis duration. With these modifications, in addition to the autoradiograms of only partially lysed polytene chromosomes, many typical examples of isolated DNA fibre autoradiograms were obtained.

Polytene chromosomes contain two distinct types of active replicons

Two distinct patterns of labelled arrays (Figs. 1–3) were seen in the polytene DNA fibre autoradiograms. The first type (Figs. 1a–d and 2) was characterized by long, nearly straight, tandem arrays of many densely labelled (hot pulse) tracks, each flanked by less densely labelled (warm pulse) tails. The length of the arrays of this type ranged from a few to several thousand µm, e.g., the parallel arrays in Figure 1a are over 6,000 µm long. In contrast, the second type was characterized by groups of irregularly arranged, shorter (on average 50 µm) arrays each containing a few tandemly placed, short, densely labelled tracks which were sometimes flanked by warm tails (Figs. 1e and 3). These two categories of labelled arrays are referred to as type I and II, respectively. The type I arrays were seen in all prepa-

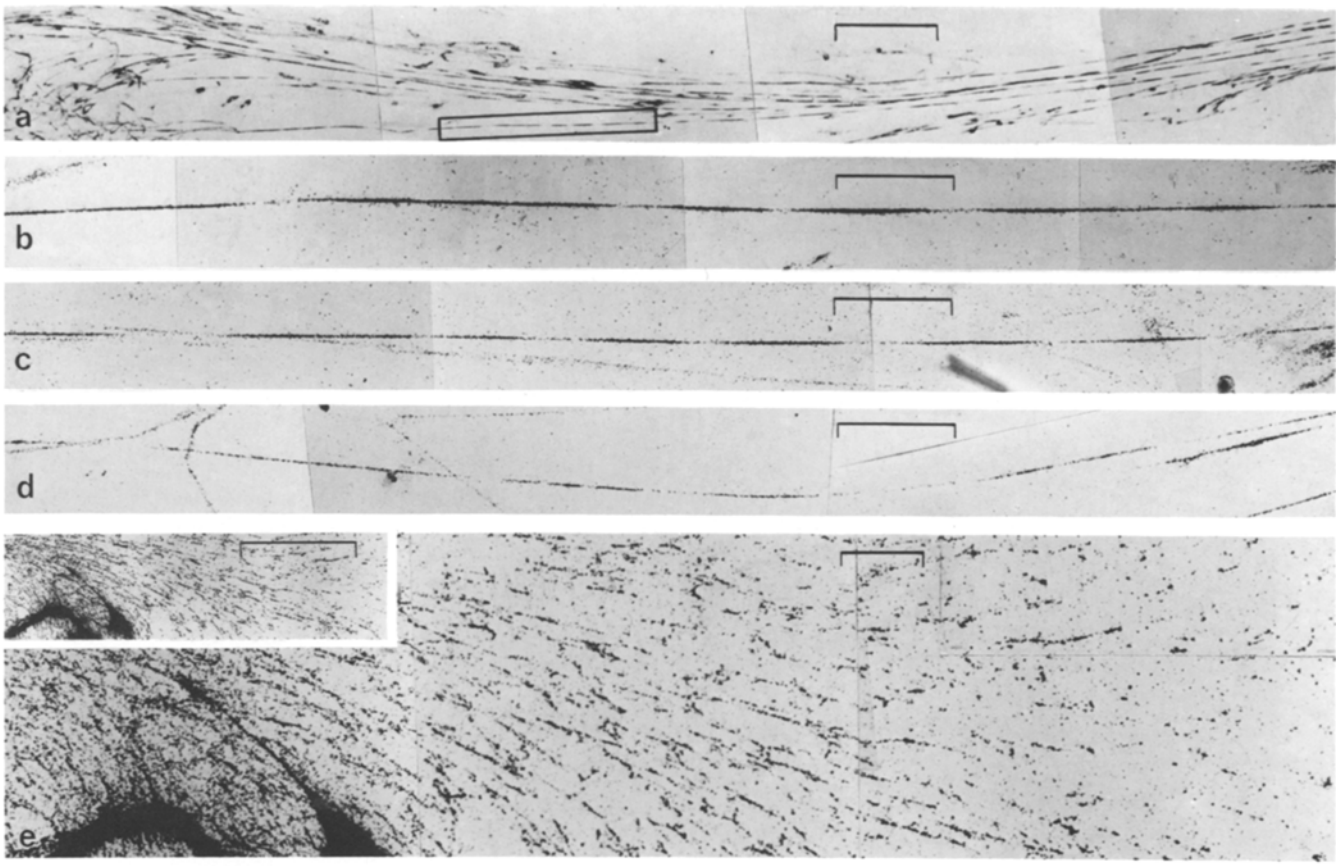

Fig. 1. Two types of active replicons in polytene chromosomes. **a** Long (more than 6,000 μm) parallel arrays of very thick type I replicons. Part of the lowermost array (*boxed*) of this group is shown at higher magnification in **b**. Note the longer dense tracks in the very thick (**b**) and the medium thick (**c**) type I arrays than in the thin (**d**) type I array. **a–d** are from 20 min-hot (+20 min warm)-pulsed synchronized glands. **e** shows groups of active type II replicons in a 60 min hot-pulsed unsynchronized gland. *Inset* shows the same field at the same magnification as in **b–d** for comparison. Note the much shorter dense track and replicon size in the type II than in type I arrays and the distinct linear alignment of the tracks in type I arrays. Bar represents 500 μm in **a**; 100 μm in **b**, **c**, **d** and in *inset* of **e**; and 20 μm in **e**

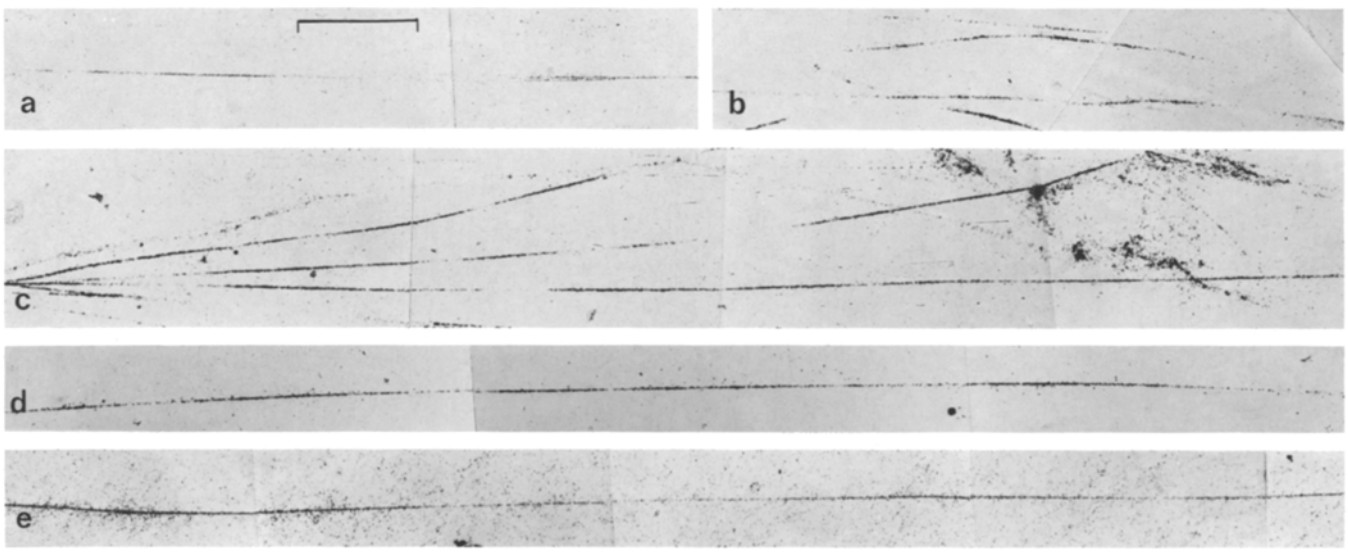

Fig. 2. Thin type I arrays from 10 min (**a**) and 60 min (**e**) hot-pulsed unsynchronized glands and from 10 min (**b**), 20 min (**c**) and 30 min (**d**) hot-pulsed synchronized glands. The warm-pulse duration was 50 min except for in **c** where it was 20 min. Note the nearly complete labelling of the arrays in **c** and **d**. Each of these labelled arrays was much longer than shown here. Bar represents 100 μm

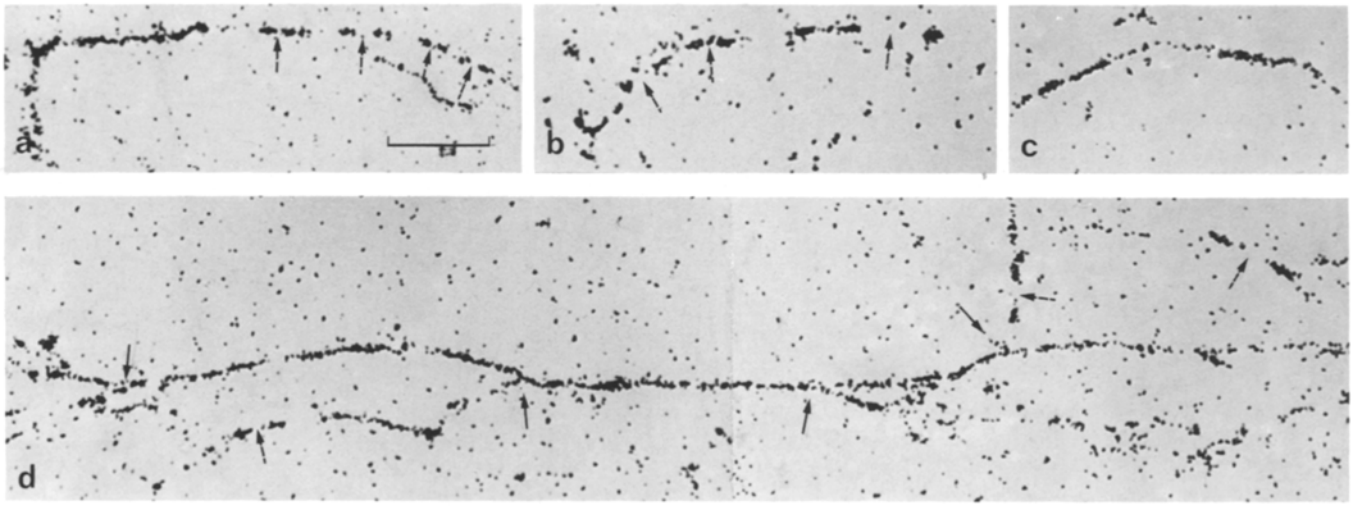

Fig. 3. Type II arrays in 60 min hot-(+50 min warm-) pulsed unsynchronized glands. **a** and **b** Groups of active replicons several of which are without warm tails (*dashed arrows*). **c** Two active replicons with flanking warm tails. **d** An unusually long type II array with a completely dense labelled central segment which on either side appears to split repeatedly (*arrows*) into tandem arrays of short dense and warm labelled tracks. This repeated forklike arrangement of the labelled type II tracks is strikingly similar to that seen in type I arrays in Figure 7. Note the nearly equal grain density of the dense tracks in the central and the split regions of the array. Some replicons (*dashed arrows*) without warm tails are also seen. Bar represents 20 μm

Table 1. High density track length, rate of fork migration and O–O distance in DNA fibre autoradiograms of different salivary gland samples

Array type	Glands	Hot pulse (min)	Mean track length ±S.E. (μm)	Average rate of fork migration (μm/min)	Mean O–O distance ±S.E. (μm)
Type I (thin)	Unsynchronized	10	8.86±0.48 (113)	0.89	67.05± 6.51 (81)
Type I (thin)	Unsynchronized	60	57.78±5.93 (33)	0.96	196.28±35.25 (24)
Type I (thin)	Synchronized	10	11.25±0.73 (108)	1.13	61.51± 4.33 (78)
Type I (thin)	Synchronized	20	21.06±0.90 (217)[a]	1.05	112.50± 4.48 (191)[a]
Type I (thin)	Synchronized	30	31.01±2.20 (95)	1.03	127.50± 8.37 (70)
Type I (medium thick)	Synchronized	20	27.75±1.07 (255)[a]	1.39	124.45± 4.26 (210)[a]
Type I (very thick)	Synchronized	20	35.39±1.53 (115)[a]	1.77	123.74± 5.03 (98)[a]
Type II	Unsynchronized	60	4.49±0.24 (123)	0.07	20.31± 1.08 (84)

Numbers in parentheses indicate sample size

[a] The mean track lengths in very thick and medium thick type I arrays in 20-min synchronized glands are significantly higher ($P<0.05$) than in corresponding thin arrays; the mean O–O distances in these samples do not differ

rations, whereas the type II arrays were present only in preparations from unsynchronized (60 min hot-pulsed) glands. The low density tails flanking the high density tracks in all type I and most type II arrays (Figs. 1–3) provide evidence for bidirectional replication (Callan 1972).

The width of the densely labelled tracks in different type I arrays varied (see Fig. 1 b–d). They have been designated as very thick (Fig. 1 b), medium thick (Fig. 1 c) and thin arrays (Fig. 1 d). The width of the dense tracks in our thin arrays corresponded to that usually seen in DNA fibre autoradiograms of diploid cells. The very thick and medium thick tracks presumably represent oligotene bundles of closely associated, parallel strands whereas the thin arrays are presumed to represent single replicating DNA molecules (also see Taylor 1977). This interpretation finds support in the electron microscopic observations on the hierarchy of constituent strands in a polytene chromosome. The many parallel strands constituting a polytene chromosome are organized into oligotene bundles of fibres (Sass 1980). This structural feature may cause some or many of these subunits to remain bound together during cell lysis thus generating thicker arrays in the autoradiograms. We selected the thin arrays for our measurements and unless specifically mentioned, type I refers to the thin arrays only. In preparations of FdUrd-synchronized glands, pulse labelled for 20 min, the medium and very thick type I arrays were also analyzed for comparison with the thin arrays. Comparable thick type II arrays were not seen.

The mean replicon lengths, lengths of the high density tracks formed by single fork during the hot-pulse period, and the rates of fork migration in type I and II arrays seen in unsynchronized and synchronized glands are presented in Table 1. Their respective frequency distributions are presented in Figures 4–6. For unknown reasons the fibre preparations from 20 min hot-pulsed unsynchronized glands did not yield many measurable arrays. Preparations of 60 min hot-pulsed synchronized glands revealed, for the most part, very long, uniformly and densely labelled, very thick or even thicker arrays and thus were not analyzable. Most type I arrays in 60-min hot-pulsed unsynchronized glands

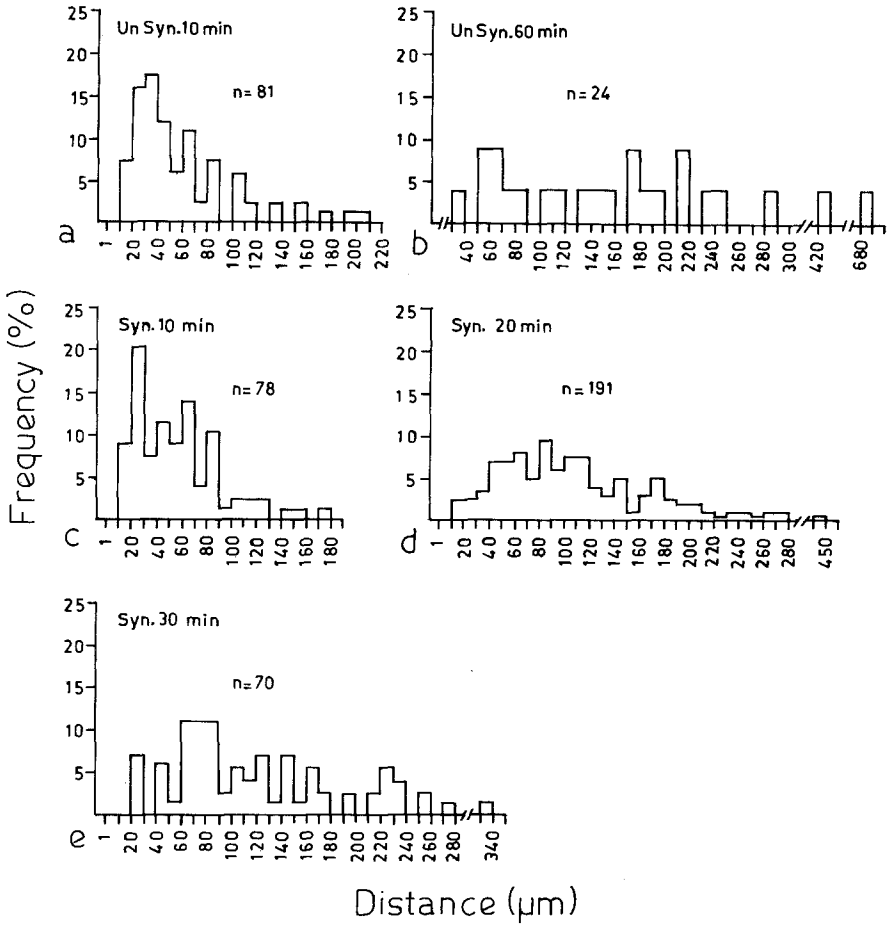

Fig. 4a–e. Frequency distributions (expressed as percentages) of origin-to-origin (O–O) distances on the thin type I labelled chromosomal DNA fibres from unsynchronized (UnSyn.) or synchronized (Syn.) salivary glands pulse labelled (hot) with ^{3}H-thymidine as indicated

were also uniformly and densely labelled for much of their length, but a few thin arrays with alternating dense and warm tracks could still be scored in this sample.

Type I replicons are long and fast replicating

From the examples in Figure 2 and the data in Table 1 and Figures 4 and 5, it is obvious that the type I arrays in unsynchronized and synchronized samples were similar with respect to replicon lengths and rates of fork migration. In all the samples, the frequency distributions of O–O distances showed more than one modal value (Fig. 4a–e), and interestingly, even with the shortest pulse period (10 min), a wide range of replicon sizes was registered (10–205 μm, see Fig. 4a, c). However, with a 10-min pulse, 75% of the replicons were in the shorter classes (less than 70 μm); whereas with longer pulses (20–60 min, see Fig. 4b–e) the larger size classes (above 100 μm) were more frequent. This increase in length with increasing pulse duration is likely to be due to fork fusion; therefore, the replicon size estimated from the shortest pulse duration has been considered to be the best approximation of the type I replicon size. In this context it may be noted that the frequent occurrence of 3C-type polytene chromosome labelling with a 10-min pulse (see Rodman 1968; Steinemann 1981b; Mishra and Lakhotia 1982a) also provides evidence for activation of the maximum possible number of origin points within the 10-min pulse period. The frequency distributions of O–O distances of type I arrays in 10-min-pulsed unsynchronized and synchronized samples (Fig. 4a, c) were positively skewed, with median values of 49.9 μm and 44 μm, respectively. The mean type I replicon lengths in these two samples (67 μm in unsynchronized and 61 μm in synchronized glands, see Table 1) were also very similar. The grand mean (64 μm) of these samples, has, therefore, been considered to be the average replicon size in type I arrays.

Comparison of the frequency distributions of the high density track lengths (calculated for single fork) in 10-min-pulsed samples of unsynchronized and synchronized glands revealed a unimodal distribution (Fig. 5a, c). With increasing pulse duration (Fig. 5b, d, e), longer track lengths are seen distributed over a wider range. The average rates of fork migration in different pulse protocols, however, were similar (ranging from 0.89 to 1.13 μm/min, see Table 1). Therefore, on the basis of the slope of the regression line for these measurements (Steinemann 1981a, b), the average rate of fork migration in type I thin arrays was estimated to be 0.95 μm/min (Fig. 5f).

The mean high density track length and the estimated rates of fork migration in medium and very thick arrays from 20 min-pulsed synchronized glands were progressively higher than in the corresponding thin arrays (see Table 1 and Fig. 1). Significantly, however, the O–O distances in these medium and very thick arrays were similar to those in thin arrays from 20 min-pulsed glands (see Table 1). A prepulse origin gap was seen in a very few thin type I arrays. None of the medium and very thick type I arrays showed a prepulse gap (Fig. 1).

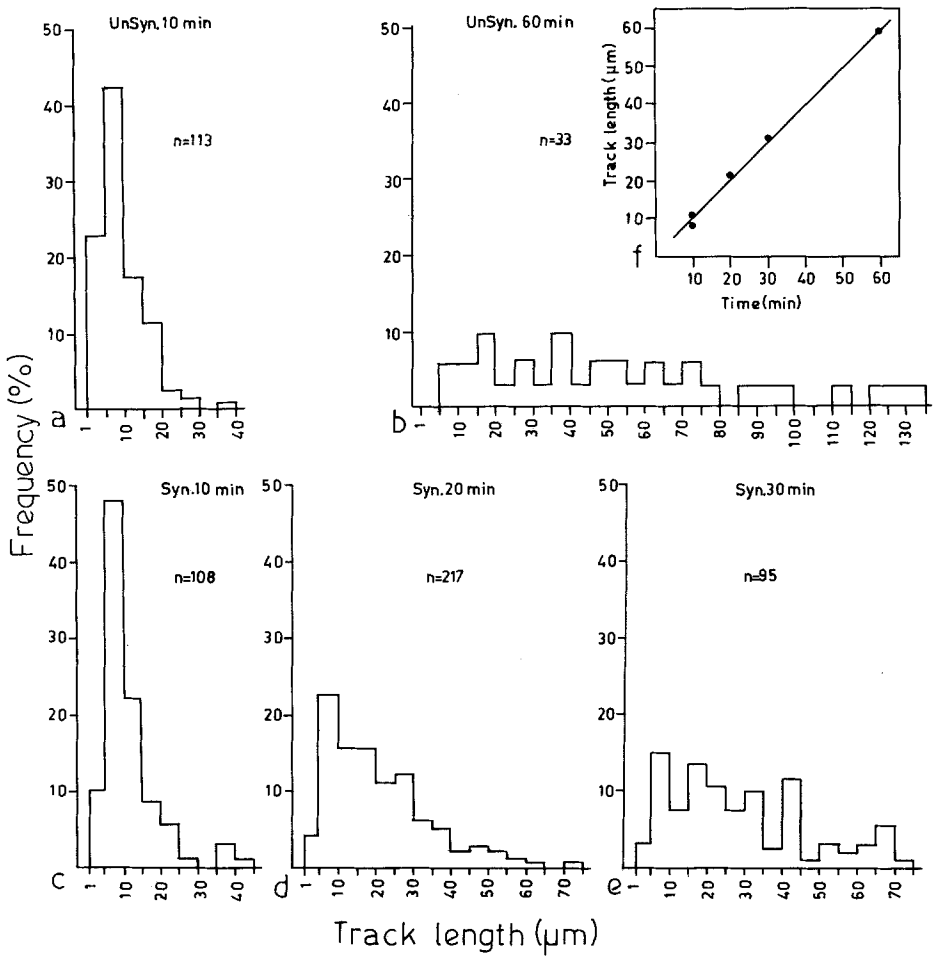

Fig. 5a–e. Frequency distributions (expressed as percentages) of high density track lengths in the type I chromosomal DNA fibres of unsynchronized (UnSyn.) or (Syn.) salivary glands pulse-labelled (hot) with ^{3}H-thymidine as indicated. **f** Average track length (Table 1) as a function of the ^{3}H-thymidine pulse duration; the slope of the regression line is 0.95. In the above, the track lengths are shown for a single fork: in arrays with a central prepulse gap, the average length of the two tracks flanking the gap was considered; in those without a central prepulse gap, the length of the DNA replicated by a single fork was calculated by dividing the measured length by 2

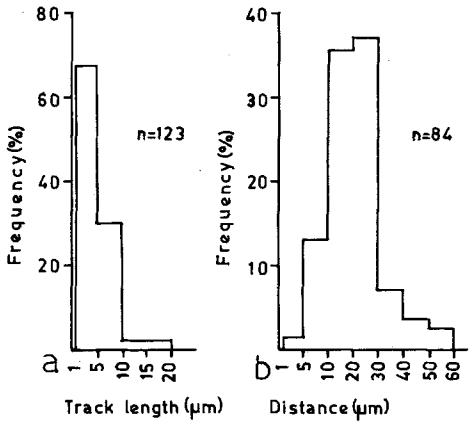

Fig. 6. Frequency distributions (expressed as percentages) of high density track lengths (**a**) and origin-to-origin distances (**b**) on the type II chromosomal DNA fibres of unsynchronized larval salivary glands pulse-labelled (hot) for 60 min

Type II replicons are short and slow-replicating

The type II arrays (Fig. 3) measured from 60 min hot-pulsed unsynchronized glands revealed a considerably slower average rate of fork migration (0.07 μm/min) and much shorter mean replicon length (20.3 μm) than the type I arrays (see Table 1 and Fig. 6). Unlike the type I arrays, both replicon and track lengths in the type I arrays showed a narrow-ranged and unimodal distribution (Fig. 6). In shorter pulse preparations the type II arrays were rarely identifiable because of the shortness of the labelled tracks, and no measurements could be made from such samples (see also Steinemann 1981b). The type II tracks were not interspersed with tandem arrays of type I tracks or vice-versa. However, the two types of arrays could be seen in one preparation of an unsynchronized salivary gland. Prepulse origin gaps were frequent in type II arrays (Fig. 3) although in most (98%) type II autoradiograms, the prepulse gap length was less than 10 μm. In some of the type II arrays, several (Fig. 3a, b) or a few (Fig. 3c, d) high density tracks were not flanked by less dense warm tails.

Labelling patterns in parallel polytene DNA fibres

Many examples of two or more closely placed, parallel type I arrays were seen in these DNA fibre autoradiograms (see Fig. 7). It is very likely that such parallel labelled arrays were due to an incomplete separation of the chromatids of a polytene chromosome. Their analysis, therefore, provides information on the progression of replication in sister chromatids.

The example in Figure 7a shows a closely aligned pair of medium thick and thin type I arrays from a 20 min hot-(+20 min warm-) pulsed synchronized gland. At the regions marked I and II, the two arrays are fused, whereas along the rest of their length they are parallel. This, taken together with the generally similar number of dense tracks and their relative locations in these two parallel arrays, suggested that the autoradiograms represent homologous regions of polytene chromatids. A closer examination (see

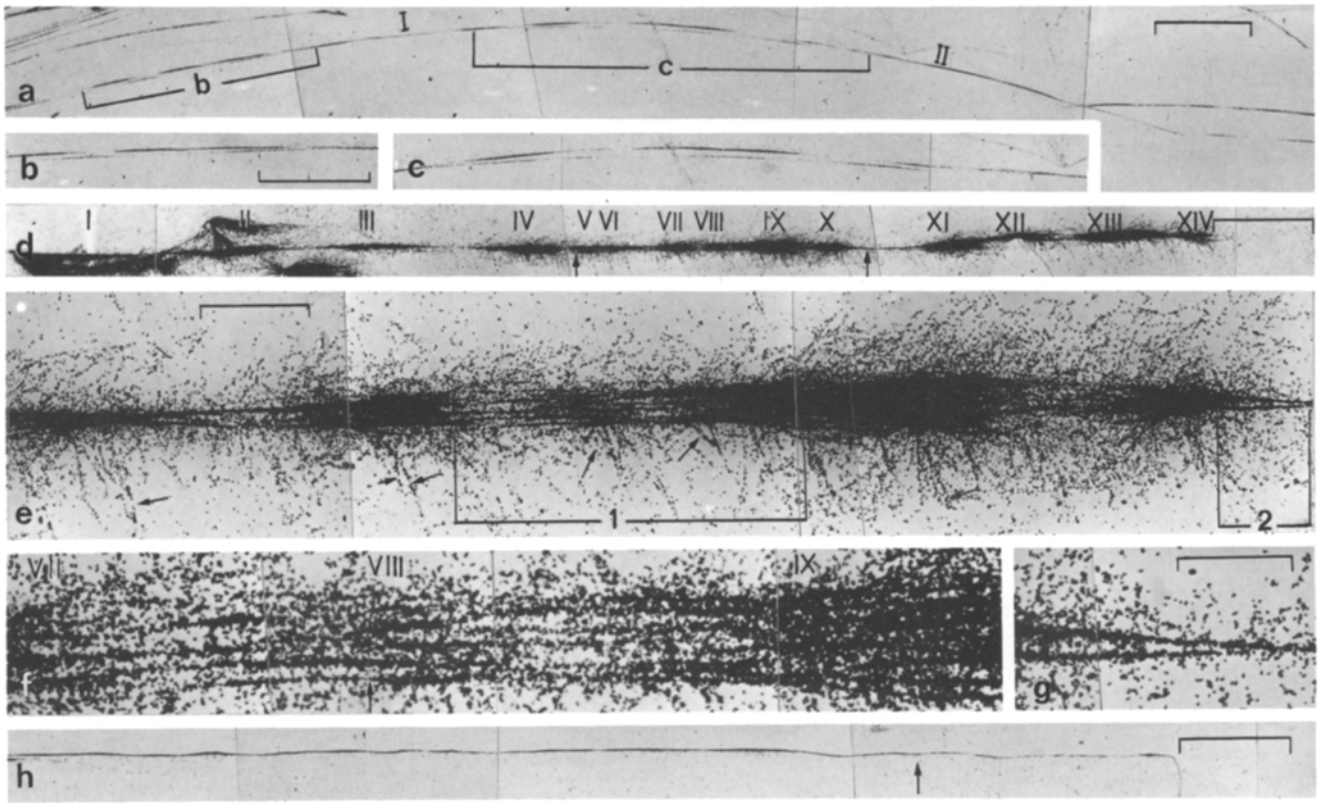

Fig. 7. Differences in time and place of activation of origin points in homologous polytene regions and laterally variable number of labelled arrays in different regions. **a** A closely aligned pair of parallel medium thick and thin type I arrays, presumably derived from homologous polytene chromatids of a 20 min hot- (+20 min warm-) pulsed synchronized gland. In regions marked *I* and *II*, the two arrays are fused together. Two *bracketed* areas of these parallel arrays are magnified in **b** and **c**, to show that many dense tracks on homologous regions of the two arrays differ in size and location. **d** Autoradiograph of a partially lysed chromosome segment from 60 min hot- (+50 min warm-) pulsed unsynchronized gland showing large variations in the number of axial tracks (type I) within and between the different densely labelled regions (*I–XIV*). The area between the two *arrows* (in **d**) shown at a higher magnification in **e** illustrates the loop-like short arrays around the central axial arrays. Some of the putative type II replicons in these short loops are indicated by *arrows*. The regions marked 1 and 2 in *brackets* are shown at a still higher magnification in **f** and **g**, respectively, to reveal the arrangement of axial tracks in part of the dense areas *VII, VIII* and *IX* (**f**) and the fork region on the right side of area *X* (**g**). **h** The type I array from a 60 min pulsed unsynchronized gland is medium thick over most of its length but thin near its right end (*arrow*). Bar represents 300 μm in a and d; 200 μm in b and h; 50 μm in e; and 20 μm in g

Fig. 7b, c), however, revealed that some of the presumptive homologous dense tracks on these two arrays differed in size or relative location. Such differences between the parallel arrays were more striking in autoradiograms of less extensively lysed polytene chromosomes (Fig. 7d–g). This autoradiograph of a lysed polytene chromosome segment was from a 60 min hot- (+50 min warm-) pulsed unsynchronized gland and extended more than 4,000 μm (partly shown in Fig. 7d). At higher magnifications (Fig. 7e–g), several (numbers varying at different positions, see below) parallel hot and warm tracks along its long axis and numerous short tracks at approximate right angles on both sides of the axis could be resolved. The disposition of the short tracks gave the impression of being arranged as numerous lateral loops (see Fig. 7e). Whereas the bulk of the lateral looplike labelling was as warm tracks, a few dense tracks (arrows in Fig. 7e) were also seen. Since in most of these cases, only two dense tracks with a small prepulse gap appeared aligned, the O–O distances in these lateral loops could not be measured. However, measurement of track lengths in 11 pairs of such dense tracks in the lateral loops revealed that in their average rate of fork migration (0.08 μm/min) they resembled the type II arrays. The O–O distances and the rate of fork migration in the axial tracks were similar to those of type I arrays. The O–O distances of the axial tracks were measured by taking the centre point of each dense cluster as the origin. The average O–O distances estimated this way was 203 μm. Eventhough such measurements were at best approximate, the estimate was within the range of type I arrays. It was difficult to precisely estimate the rates of fork migration on the axial tracks, but an approximation of 0.3 μm/min was obtained by measuring the length of the few distinctly resolvable parallel dense tracks of the type seen in cluster VIII in Fig. 7f. On the basis of these observations we suggest that in this and other similar (not shown here) autoradiograms of partially lysed chromosomes, the axial tracks were formed by type I replicons whereas the lateral loops were formed by type II replicons.

The pattern of straight-running hot and warm tracks, the regular spacing of the parallel axial arrays and the presence of dense tracks in generally similar locations (clusters I to XIV in Fig. 7d) on the parallel arrays suggested that the underlying DNA fibres of a polytene chromosome subunit had unfolded and stretched but retained their in vivo parallel alignment (see Discussion). At higher magnifica-

tions (Fig. 7f–g), however, it was clear that the densely labelled tracks on the parallel arrays in a given cluster (as in cluster VIII) differed from each other in relative location and length. The other interesting feature of the autoradiograph of the type shown in Figure 7d–g was the large variation in the number of parallel axial arrays and the variation in the thickness of the densely labelled tracks along the length of an array. The lowermost array in cluster VIII shows hot tracks of unequal thickness on both sides of the putative origin (arrow in Fig. 7f). Interestingly, the thin dense track on the left side continues in cluster VII as a single warm labelled track, whereas the thicker dense track on the right appears to bifurcate before entering cluster IX. If one follows the bunch of axial tracks in cluster VIII to the right into the dense cluster IX, many of them appear to branch further so that within cluster IX in Fig. 7f there are many more parallel axial tracks than in clusters VII or VIII. Following these labelled arrays further to the right into cluster X or beyond, it is seen that in this region the parallel labelled tracks progressively merge until they appear as two "prongs" of a single fork (see Fig. 7d, g) which again split into two and more arrays in the dense cluster XI on the right (Fig. 7d).

The above type of unequal thickness of a dense track along an array or the splitting of a track into two or more arrays was also sometimes seen in more distinctly isolated arrays. The type I array in Figure 7h extended over more than 2,000 μm and was medium thick over most of its length, but at its right end (arrow) it tapered into a typical thin-type array. The type II array in Figure 3d also shows a repeated branching of the labelled tracks. The grain densities in the single and branched regions in such arrays (Figs. 3d, 7g) were similar.

Discussion

A basic difficulty in DNA fibre autoradiography of polytene chromosomes is the lateral association of their many parallel DNA fibres. Unless they are effectively separated, the autoradiograms may give erroneous results. We believe that the strong pronase treatment coupled with a longer period of exposure to SDS-EDTA employed by us would have caused the constituent polytene chromatids to be well separated. In our autoradiograms we observed labelled arrays of different thicknesses reflecting different degrees of separation of the bundles of polytene fibres. However, since the thin arrays analyzed by us are comparable in their width to those found in similar arrays of typical diploid nuclei, we feel justified in considering our thin arrays to represent single replicating polytene chromatids.

Our observations suggest the existence of two remarkably different categories of active replicons in polytene nuclei of *D. nasuta*: the type I replicons are, on average, more than three times longer (64 μm) and ten times faster (0.95 μm/min) than the type II replicons. The following considerations make it unlikely that these two categories of replicons are extremes of a single type: (1) the replicon types I and II were never aligned on the same labelled array; (2) the tye I usually occurred in large tandem clusters, whereas the type II were always in much smaller arrays; (3) comprison of the track lengths and O–O distances in the type I and II arrays from 60 min unsynchronized glands (Figs. 4b, 5b, 6a, b) revealed negligible overlap between the two types. Those few tracks that were similar in track length differed in O–O distance and vice-versa. The presence of type I and the absence of type II arrays in FdU-synchronized glands also suggests that the two types of replicons are different.

Unlike the earlier report in CHO cells (Taylor 1977), FdUrd blockage in polytene nuclei did not result in activation of a larger than normal number of origin points since O–O distances in the type I arrays from unsynchronized and synchronized glands were similar (see Figs. 2, 4, 5). At the chromosomal level too, the number and location of the early replicating sites in the FdU-synchronized glands were similar to those in the unsynchronized glands (P. Sinha, unpublished). Thus, we believe that the FdU treatment did not cause any alterations in the replicative organization of polytene nuclei.

On the basis of the average DNA content estimated for one band (~10 μm, Beermann 1972; Rudkin 1972), the average type I and II replicons correspond to about six and two band DNA lengths, respectively. Thus, the earlier concept of one band as one replicating unit is not valid (see also Steinemann 1981b). The type I replicons seen in polytene nuclei resembled those reported by Blumenthal et al. (1973) in cleavage nuclei of *D. melanogaster* with respect to their clustering in large tandem arrays and their high rate of fork migration. The considerably higher replicon size (type I) in larval salivary glands than in embryonic cells (Blumenthal et al. 1973) conforms to the general reduction in number of active origin points in diferentiated cells (Callan 1972; Blumenthal et al. 1973). The presence of two distinct kinds of replicons in the same cell type is most intriguing. A comparable situation has not been seen in any other cell type of *Drosophila* (Blumenthal et al. 1973; Ananiev et al. 1977; Steinemann 1981a, b) or of other eukaryotes (Hand 1979). Presumably this extraordinary feature is related to the unique organization of polytene chromosomes.

Early and late S replicons

The following evidence suggests that the type I and II replicons are active in early and late S periods, respectively. In DNA fibre autoradiograms of FdUrd-synchronized glands of *D. nasuta* larvae in which nearly all the replicating polytene nuclei show early S (IB) patterns of chromosomal labelling (Sinha and Lakhotia 1983; see also Achary et al. 1981), only the type I replicons are active. We correlate the selective absence of type II replicons in synchronized glands to the absence or rare occurrence of the late D-labelled nuclei in glands pulse-labelled immediately after release from FdU block. In unsynchronized glands on the other hand, the presence of active type I and II replicons can be correlated with the presence of early as well as late S nuclei. The spatial distribution of label observed at chromosomal level in early and late S nuclei of *D. nasuta* (Roy and Lakhotia 1981) further supports the above view. In very early S when chromosomes show the IB-labelling pattern, ^{3}H-thymidine incorporation is seen mainly in interband and puff regions. The average spacing of these labelled chromosomal sites (five to six bands, see Fig. 9b) is comparable to the average O–O distance (~64 μm) in the type I arrays. Moreover, corresponding to the large number of replicating sites in the IB- and C-labelled chromosomes (see Fig. 9b–d), the type I arrays show a long tandem series of active replicons. Thus, we suggest that the origin points

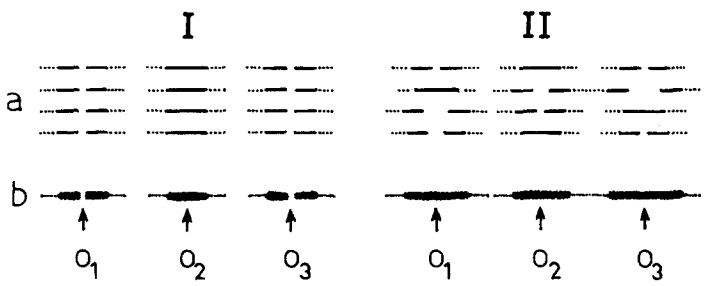

Fig. 8. Lateral strand asynchrony and the observed properties of the thick type I labelled arrays. The medium and very thick type I arrays are formed by tight association of a number of labelled polytene chromatids. In the schematic diagram here, four parallel strands (**a**) are presumed to remain together to generate a medium thick type labelled array in the fibre autoradiogram (**b**). In *scheme I*, the homologous origin points (O_1–O_3) on all parallel strands are presumed to be activated at the same time; in *scheme II*, they are asynchronously activated (for simplicity, spatial differences in the location of origin points on parallel strands are not considered, see Figure 9). The *solid* and *dotted lines* indicate the regions labelled during hot and warm pulses, respectively. With temporal synchrony on all polytene strands, the thick type labelled arrays will show the same lengths of densely labelled tracks as the thin arrays (*scheme I*) while with temporal asynchrony in homologous polytene origin points, the thicker arrays would show a longer densely labelled track (and hence a higher estimate of the rate of fork migration) than the thin arrays (*scheme II*). The O–O distances will be the same in all cases but the prepulse gaps are obscured in *scheme II*. The observed properties of the thin and thicker type I arrays are in accordance with those in *scheme II*

of type I replicons are located in the early S-labelled interband/puff regions (Fig. 9). The late replicating sites, each consisting of one or a few bands, are widely spaced as seen in 2D- or 1D-labelling patterns (Arcos-Terán 1972; Rudkin 1972); these sites in DNA fibre autoradiograms would, in general, correspond to the small groups of shorter replicons seen in type II arrays. Autoradiograms of partially lysed polytene chromosomes, which could be compared with late S discontinuous (2D or 1D) labelling patterns, were always associated with type II but not type I arrays. These also support the association of type II replicons with late S replication. The very slow rate of fork migration in type II replicons appears to contribute to the longer duration (see Introduction) of the D-labelling patterns. DNA fibre autoradiographic studies using glands in which only the D-labelling is seen, are in progress to confirm the association of the type II replicons with late S.

In an earlier DNA fibre autoradiographic study of polytene nuclei of *D. virilis* only one class of replicons was noted (Steinemann 1981b), which, in general, resembles the type II replicons in our study. The difference in average replicon size estimated by Steinemann (46.7 μm) and that estimated by us (20.3 μm) for the type II arrays is partly related to their being estimated from 120- and 60-min pulses, respectively. The absence of the type I replicons in the samples analyzed by Steinemann (1981b) may be due to the method of cell lysis. Moreover, during the longer hot-pulse periods (60 and 120 min), analyzed by Steinemann (1981b), fusion of most of the tandem type I replicons is expected. This would result in a continuous labelling of these fibres without distinct hot and warm tracks. In our 60-min hot-pulse preparations also, most of the type I tracks were of this type. Such uniformly labelled tracks are not amenable to analysis.

Asynchronous replication of polytene chromatids

The differences in size and relative location of the putative homologous densely labelled tracks in the parallel arrays (Fig. 7) suggest that the origin points in homologous replicons of lateral strands are activated at different times and locations. Comparison of the properties of medium and ery thick type I arrays with those of thin arrays also suggests an asynchronous replication of homologous replicons on lateral strands. As illustrated in Figure 8, the increase in track size with increasing thickness of the labelled arrays, but nearly identical O–O distances in the thin and thicker arrays (see Table 1, Fig. 1) can be explained by assuming that while the fibres constituting the bundle remain laterally aligned, the homologous origins in them are activated asynchronously. The increase in the length of dense tracks without any change in O–O distance in the thicker arrays could also be generated by lateral slippage of the constituent parallel fibres of a polytene subunit during lysis. However, the following considerations argue against this possibility. First, medium and very thick arrays over several thousand μm long (see Fig. 1a) have been frequently observed, and all along their length they show a very regular pattern of dense and warm tracks. Since the constituent fibres are not rigid rods, they cannot possibly slide uniformly over such long lengths. Second, arrays of the type seen in Figures 3d and 7a–g often show the dense tracks on the parallel arrays to be not only relatively "displaced" but also of different sizes. While slippage might cause a displacement of the dense tracks, it would not alter their sizes. In this context differential stretching of the DNA fibre could also be a source of artifact. However, considering the following points we believe that as in any other DNA fibre autoradiographic study, differential stretching is not a serious problem: (1) the thicker as well as the thin type I labelled arrays run fairly straight and appear reasonably well extended with a uniform thickness along their entire length. If some regions of the underlying DNA fibres were folded (less stretched), the corresponding autoradiograms would be thicker than the more stretched regions. (2) If the thin type I arrays are more stretched than the thick and very thick type I arrays, the track lengths (rate of fork migration) estimated for the thin arrays should be higher than those for thicker arrays. However, the estimated rates were actually higher the thicker the array (see Table 1). Several other considerations discussed below also favour asynchronous replication on parallel polytene chromatids.

As in other differentiated cells (Callan 1972; Blumenthal et al. 1973; Taylor 1978), there are many more potential origin points than are actually used in salivary gland polytene nuclei. The activation of one of the many potential origin points in differentiated cells has been suggested to be a probabilistic event depending upon a certain minimum local concentration of initiation factors (Taylor 1977, 1978). The large number of lateral polytene chromatids would accentuate the competition for these initiation factors. Thus, the locations of the activated origin points and their time of activation in a given chromosomal replicating site are likely to differ between polytene strands unless additional mechanisms are invoked to identify and simultaneously activate a specific origin point on all polytene strands. The logistics of accomodating the large number of DNA polymerase and other enzymes or factors needed for replication may also preclude a simultaneous activation of homologous

origin points on all the polytene copies of a replicon. It should be noted that the commonly held belief of synchronous replication of all polytene copies of band DNA (Rudkin 1972; Steinemann 1981 b) is based on identical labelling of asynapsed homologs. However, the chromosomal patterns of labelling define only the overall periods of replication of a given chromosomal site, and do not necessarily imply an absolute synchrony in initiation and termination of replication in the lateral chromatids. Furthermore, when the labelling patterns of specific polytene chromosome regions in different nuclei are arranged in matrices of ordered arrays, many sites show "exceptional" or "unexpected" presence or absence of labelling (Rudkin 1972). Such exceptional labelling patterns may be due to a lack of temporal and spatial agreement in replication of lateral strands (see Mishra and Lakhotia 1982 b).

Lateral strand asynchrony explains different polytene chromosome labelling patterns

Further support for asynchrony in replication of parallel strands is provided by two basic features of polytene replication. The first is the frequent occurrence of continuous or C-labelling and the second is the gradual but significantly large increase in the amount of ^{3}H-thymidine incorporated at a given chromosome site as the nucleus passes from low to heavy interband and C-labelling stages and a comparable degree of decline in the labelling density from 3D- to 1D-labelling stages (Rodman 1968; Kalisch and Hägele 1973; Roy and Lakhotia 1981). As discussed above, the faster replicating (0.95 μm/min) type I replicons with an average spacing of 64 μm DNA between origin points in the interband regions, are active in the early S when C-labelling is observed. During a 10-min pulse of ^{3}H-thymidine only the middle third, i.e., about 20 μm will be replicated on average. Thus, with synchronous replication of all polytene chromatids, some regions would always remain unlabelled after a 10-min pulse. However, C-labelling is observed with as brief a pulse as 1 or 2 min (Mishra and Lakhotia 1982 a). Even if one assumes that in the C-labelling phase all type I and II replicons are synchronously active, the uniform labelling characteristic of this stage cannot be generated in autoradiograms of chromosomes labelled with brief pulses. The large increase in incorporated radioactivity at a given chromosomal site during the early S and the decrease during the late S would imply, if all polytene chromatids were replicating synchronously, a corresponding increase or decrease in the rate of fork migration. Neither our data nor any other in the literature (Yurov 1979) provide evidence for such large differences in the rate of fork migration in a single replicon at different stages of its replication. As illustrated in Figure 9 the lack of temporal and spatial agreement in initiation and consequently in progression of replication in lateral polytene strands satisfactorily explains both these aspects of polytene chromosome replication. The large decline in the grain density over a given late-replicating site in the D-labelled nuclei also appears to be due to a similar lateral asynchrony in the activation of type II replicons.

The degree of synchrony of replication in homologous replicons would thus be one of the determinants of the total time in which a given polytene chromosome site completes its replication cycle (= labelling frequency in chromosome autoradiograms) and the silver grain density seen over the site in different patterns of labelling (= rate of replication at the chromosomal level). In this context, the faster completion of replication of the hyperactive X in male polytene nuclei (Lakhotia and Mukherjee 1970) appears to be due to a greater synchrony of its parallel strands. This increased synchrony is probably due to its hemizygosity (half the number of active origin points and thus less competition for the initiation factors) and a looser packing (facilitating precursor and polymerase supply) of its constituent chromatids required for the increased rate of transcription in connection with dosage compensation (Mukherjee and Beermann 1965).

Local variations in polyteny

The observed unequal numbers of labelled type I tracks in the incompletely separated polytene chromatids and their frequent forklike arrangement in the autoradiograms (Figs. 3 d, 7 d–g) raise the possibility of variation in levels of polyteny of different chromosomal regions (Sorsa and Virrankoski-Castrodeza 1976; Roberts 1979; Cowell and Hartmann-Goldstein 1980; Laird 1980). This disparity in the numbers of labelled tracks in the "prong" and "handle" regions of such fork-like labelled arrays (Figs. 3 d, 7 d–g) is possibly not a consequence of tight association of laterally placed replicating DNA fibres in the "handles". If that were the case, the dense track(s) in the "handles" should appear thicker than those in the "prongs". This, however, has not been seen (Figs. 3 d, 7 d–g). Therefore, we believe that there are fewer *replicating* DNA strands in the "handle" than in each of the "prong" regions of the autoradiograms. Likewise the variations in thickness of the tandem hot segments of the thicker labelled arrays (Fig. 7 f, h) are also most likely due to a similar fork-like organization except that since the "prongs" of the fork have remained closely held together in the underlying fibres, only a single array with varying thickness of the hot segment is visualized in the autoradiograms. The difference in the number of replicating molecules in the two regions could be either due to (1) asynchrony in replication of the parallel polytene DNA fibres or (2) a forklike structural organization of the underlying polytene chromatids. In the first case, it may be imagined that in the "handle" regions, some strands have already completed or not yet initiated their replication whereas in the "prong" regions the parallel strands replicate with greater synchrony. On the other hand, if different regions have variable levels of polyteny, the junction regions of lower and higher polyteny would be expected to sometimes generate fork-like labelled arrays in fibre autoradiograms. We do not favour the first possibility since the fork-like labelled arrays in Figures 3 d and 7 were seen in glands labelled for a long period of 60 + 50 min. The difference in the time of replication between the parallel polytene strands presumably is not so large. We, therefore, tentatively interpret these fibre autoradiograms in favour of local variations in the levels of polyteny along the length of a chromosome (Sorsa and Virrankoski-Castrodeza 1976; Roberts 1979; Cowell and Hartmann-Goldstein 1980; Laird 1980). However, their real significance will be understood only when the underlying fibres are visualized. Electron microscopic analysis in conjunction with DNA fibre autoradiography would be useful in this context.

Finally, although the present results provide evidence for the activity of the type I and II replicons in early and

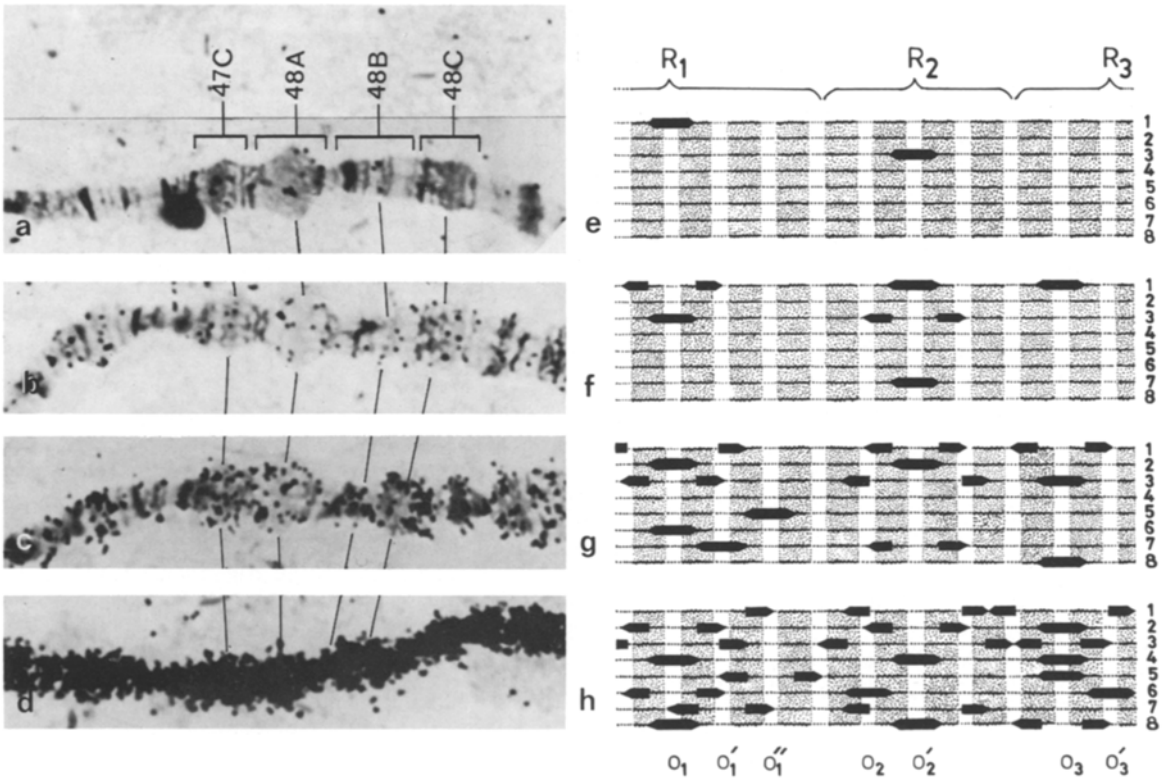

Fig. 9. Lack of temporal and spatial agreement in activation of replicons in lateral polytene strands and generation of interband and continuous patterns of chromosomal labelling. During early S more and more puffs, interbands and thin bands are labelled resulting in low interband (**a**), medium interband (**b**), heavy interband (**c**) and continuous (**d**) patterns of chromosomal labelling (Roy and Lakhotia, 1981) on a segment of 2R of *D. nasuta* after a 10-min ^{3}H-thymidine pulse. A model to explain the generation of these patterns is shown on the right (**e–h**). Only 8 parallel polytene strand (*numbered 1–8*) are considered; the DNA of bands (*stippled*) is shown as *solid lines*, that of interbands, as *dotted lines*. Bands and interbands are grouped into three replicating units (R_1, R_2 and R_3) at the chromosomal level. Within interband regions of each replicating unit, are several origin points (*O, O', O''* etc.) any one of which may be activated on a given strand (lack of spatial agreement in lateral strands). The times of activation of the origin points on the different strands in a unit also differ (temporal asynchrony). The DNA segments labelled during a 10-min hot pulse of ^{3}H-thymidine on the different strands are shown as *solid black bars*, the pointed ends of which indicate the direction of chain growth (at a rate of $\sim$1 μm/min/fork). In **e** only O_1 on strand 1 and O_2 in strand 3 are active so that at the chromosomal level low labelling of a few sites is seen (as in **a**). In their DNA fibre autoradiograms densely labelled tandem tracks will be too far apart to be identifiable as an array. In **f** and **g** which correspond to (**b**) and (**c**), respectively, more origin points, laterally as well as linearly, are activated while the forks which have been activated at the (**e**) stage migrate to adjacent sites; thus in the chromosomal autoradiograms more sites with greater silver grain density appear labelled. As more and more origin points are activated a stage (**h**) is reached when nearly every band and interband region has one or more active fork(s); such chromosomes would show continuous labelling as in (**d**). The large differences in the silver grain densities in 2C and 3C type of chromosomal labelling (Rodman 1968) can be visualized by extending the above scheme to polytene chromosomes actually containing hundreds of parallel strands rather than 8 as considered in the diagram

late S, respectively, we do not know if at some stage of polytene S, both the replicon types can be active together. Since we did not see any labelled array displaying both type I and II replicons, we assume that if the activities of these two replicons overlap at some stage of the polytene S period, they probably would be on separate chromatids. The autoradiograph in Figure 7d may be an example of this type, although with the long period of labelling (60 min hot + 50 min warm) the two types of replicons may have been active consecutively rather than simultaneously. In any case, the relationship of these two types of replicons to the possible variable levels of polyteny along a chromosome and other features of polytene replication is not yet fully understood.

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