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Replication in *Drosophila* Chromosomes : Part II—Unusual Replicative Behaviour of Two Puff Sites in Polytene Nuclei of *Drosophila kikkawai*

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Replication in polytene nuclei of late 3rd instar larvae of *D. kikkawai* has been studied by autoradiography of squash preparations of salivary glands following a 10 min *in vitro* pulse of ^{3}H-thymidine. In addition to the usual continuous (all sites labelled) and discontinuous (condensed bands and chromocentre mainly labelled) types of autoradiographic labelling patterns, a variety of interband (IB) types of labelling patterns have been seen with a relatively high frequency. The disperse regions like puffs and interbands are chiefly labelled in interband type of labelled patterns. Depending on the number and degree of labelling of these disperse regions, 3 major categories of IB patterns, viz. low (LIB), mid (MIB) and heavy (HIB) interband types have been recognized in *D. kikkawai*. The replication of 2 puff sites, 11E and 3C on arms E and C, respectively, of the reference photomap presented here, shows unusual features since these 2 sites were labelled in all kinds of labelling patterns. It is suggested that in polytene nuclei of late 3rd instar larvae of *D. kikkawai* the initiation of replication at different early replicating sites (mainly disperse regions) of a nucleus is greatly asynchronous and some of the initiating sites (e.g. puffs E-11E and C-3C) continue to replicate till later part of the 'S' period.

THE sequential and ordered replication of different replicating units in chromosomes of higher organisms has been very well demonstrated in studies on polytene chromosomes of *Drosophila*. Several recent ^{3}H-thymidine labelling studies have now provided a general picture of the sequential replication of the individual sites on polytene chromosomes of late 3rd instar larval salivary glands : the replication cycle is believed to be initiated at interbands and/or puff sites (disperse regions); the chromocentre and bands (compact regions) start replicating later and continue when the interbands and other early replicating sites have completed their cycle. Thus there is a discontinuous labelling of chromosomes both at the beginning as well at the end of the S and these 2 discontinuous types have been seen to be complementary to each other in several *Drosophila* species examined[1-5]. In the present study we have examined ^{3}H-thymidine labelling patterns in the polytene chromosomes of *Drosophila kikkawai*. The major point of interest in this species is the replicative behaviour of two puffs which apparently replicate during the entire S phase. A comparable replicative behaviour has not been reported in any other *Drosophila* species. A preliminary account of ^{3}H-thymidine labelling patterns in polytene chromosomes of *D. kikkawai* was presented earlier[6].

Materials and Methods

A wild type strain of *D. kikkawai* (from Brazil) has been used in these studies. Larvae were grown in standard cornmeal—agar medium at 20° ± 1°C, and salivary glands from late 3rd instar larvae (black spiracle stage[7]) were dissected in *Drosophila* Ringer solution. The excised glands were pulse labelled for 10 min with ^{3}H-thymidine (250 μCi/ml; Sp. act. 10.4 Ci/mM; obtained from BARC, Trombay); the labelled glands were fixed, squashed and processed for autoradiography with Ilford L4 emulsion. The coated slides were exposed in dark for 9 days at 4°—6°C and then developed with Kodak D19b, fixed, washed and air-dried. The dried autoradiograms were stained with Giemsa, mounted with DPX and examined for different labelling patterns.

To help the identification of different chromosome regions, a photomap of the polytene chromosomes of *D. kikkawai* has been prepared from temporary aceto-orcein-carmine squash preparations. Different chromosome segments were photographed under phase-contrast optics and photomicrographs of well spread segments of chromosomes joined together to make a photomap.

Results

Polytene chromosomes of D. kikkawai — Polytene nuclei of *D. kikkawai* have 5 long arms and a short 'U' shaped 4th chromosome attached to a common chromocentre. The 5 long arms represent the X-chromosome (acrocentric) and the left and right arms of the 2nd and 3rd pair of metacentric autosomes, respectively. In an earlier preliminary communication[8] we presented a photomap of *D. kikkawai* in which we designated the 5 long arms as X, 2L, 2R, 3L and 3R, respectively, and numbered the subdivisions on these five arms from 1 to 100. However, it appears that the designation of the four long arms as 2L, 2R, 3L and 3R was premature since in the absence of cytogenetic data one cannot be

certain about the left and right arms of a given chromosome in polytene nuclei where all the arms are joined to a common chromocentre. In view of this, we feel that at present it would be better to designate the different arms as A, B, C, D and E. The arm A represents the X-chromosome while the arms designated as B, C, D and E represent the four arms of the 2nd and 3rd pair of metacentric autosomes. Accordingly, we have presented a revised photomap of *D. kikkawai* polytene chromosomes in Fig. 1. In this map, each of the long arm is divided into 20 divisions, numbered 1 to 20 starting from tip to the chromcentre end. Each of the division is further subdivided into 4–6 sections, numbered alphabetically.

In the present study on replication of *D. kikkawai* polytene chromosomes, we have analysed the autoradiographic labelling patterns on two chromosome segments, namely the segment from 1A to 3D on arm C (C–1A to C–3D) and the segment from 11A to 12D on arm E (E–11A to E–12D). A detailed cytological picture of these 2 segments is given in Fig 2 where the different sections of these 2 chromosome segments have been further divided into smaller regions in relation to the analysis of ^{3}H-thymidine labelling patterns.

General patterns of labelling — A total of 1874 nuclei were examined from ^{3}H-thymidine labelled salivary glands of 31 larvae. The larvae were taken at various intervels (over a period of 24 hr) after the anterior spiracle turned black so that the overall frequency of different types of labelling patterns during the late third instar stages could be ascertained. The general autoradiographic labelling patterns of polytene nuclei seen after a 10 min *in vitro* pulse of ^{3}H-thymidine to larval salivary glands of *D. kikkawai* have been grouped into the following broad categories (also see Fig. 3). The salient features of these categories are given below, but some interesting deviations in these patterns are discussed later.

Interband (IB) tppe—Labelled nuclei of these types show a restricted labelling of interbands and puffs with most of the bands and chromocentre being either unlabelled or low labelled. These patterns correspond in general to the 'disperse discontinuous' (DD) patterns described by Chatterjee and Mukherjee[3] in *D. pseudoobscura*. The different interband patterns seen have been further subgrouped into the low (LIB), mid (MIB) or heavy (HIB) interband labelling patterns on the basis of the number and grain density of the labelled regions. In LIB patterns, a low labelling in seen mainly over 2–3 puffs and a few interbands. The MIB class consists of a wide range of labelled nuclei with more puffs and interbands labelled with a low to moderate grain density. In HIB patterns, nearly all puffs and interbands are moderately labelled. Several bands are also labelled but many remain clearly unlabelled. In LIB, the chromocentre is almost unlabelled while in MIB and HIB types, there is a progressively greater labelling of the chromocentre region.

Continuous type—Nuclei which show a nearly uniform labelling of most bands, interbands, puffs and chromocentre have been classified as continouous type. Depending on the intensity of labelling of the nucleus, 2 categories—medium (2C) and heavy (3C) continuous types, have been recognized following Rodman[9].

Discontinuous type—In these nuclei, the labelling is restricted mainly to the dark bands and the chromocentre (compact regions); again following Rodman's[9] classification, heavy (3D), mid (2D) and low (1D) discontinuous patterns have been recognized.

On the basis of the above criteria for classifying the labelled nuclei, the 1874 nuclei observed were grouped into various categories. The data are presented in Table 1. The data on the pooled frequencies of different labelling patterns of the late third instar larvae of *D. kikkawai* reveal several interesting features. The frequency of unlabelled and discontinuously labelled nuclei is very low in *D. kikkawai* during the stage of larvae used for study. The continuous patterns (specially the 2C types) are very common. However, most interesting is the relatively high frequency of the interband patterns, particularly the MIB. In the pooled data, the frequency of all types of interband patterns taken together is slightly more than 25%. In some individual cases, it was noted that the interband patterns are present in even more than 50% of all polytene nuclei present in the preparation.

Temporal sequence of ^{3}H-thymidine labelling patterns in polytene chromosomes of D. kikkawai — It is now widely believed that the different kinds of autoradiographic labelling patterns observed after a short pulse of ^{3}H-thymidine to polytene cells, relate to nuclei which are in different phases of a given polytenic replication cycle[3,10,11], and, therefore, we presume that the above noted different kinds of labelling patterns also relate to the temporal sequence of replication of polytene chromosomes of *D. kikkawai*. During our examination of the general ^{3}H-thymidine labelling patterns in *D. kikkawai* chromosomes, the patterns of ^{3}H-thymidine incorporation in a few chromosomal sites (viz. 11E and 12C on arm E and

TABLE 1 — FREQUENCY OF DIFFERENT TYPES OF ^{3}H-THYMIDINE LABELLING PATTERNS IN POLYTENE NUCLEI OF LATE THIRD INSTAR LARVAE OF *D. kikkawai*

[Abbreviations of labelling patterns as in the Text]

	Autoradiographic labelling patterns								Unlabelled	Total no. of nuclei*
	LIB	MIB	HIB	2C	3C	3D	2D	1D		
No. of nuclei	30	286	170	520	301	151	167	73	176	1874
% of total nuclei	1.6	15.3	9.1	27.7	16.1	8.1	8.9	3.9	9.4	

*Data pooled from 31 larvae

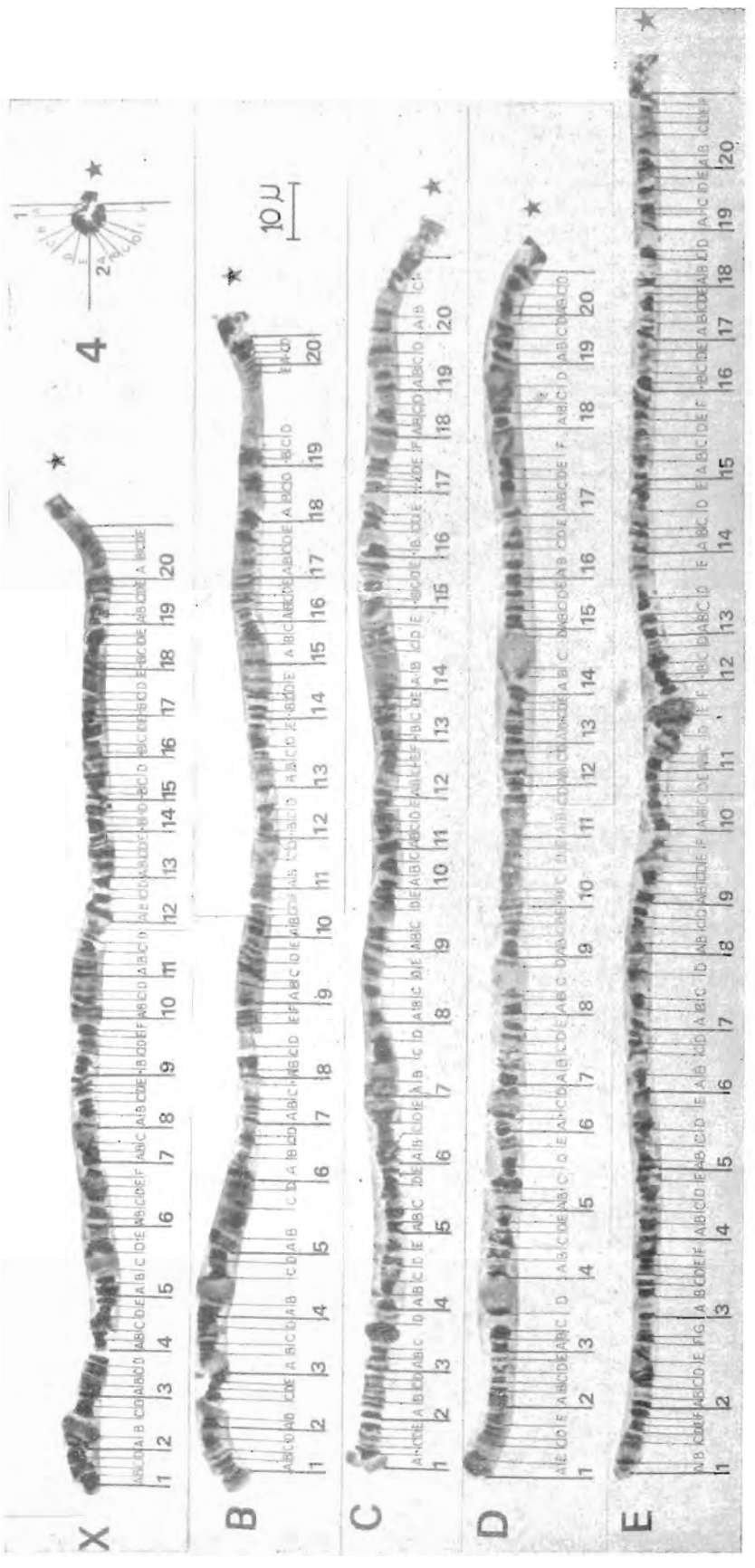

Fig. 1 — Photomap of salivary gland polytene chromosomes of *D. kikkawai*

3C on arm C) appeared to be rather unusual since these three sites were seen to be labelled in almost all labelled nuclei, except that E-12E was low labelled or unlabelled in 2D and 1D type of nuclei. Therefore, to understand the termporal sequence different labelling patterns and to analyse the unusual replication behaviour of the above mentioned 3s ites, we have examined the ^{3}H-thymidine labelling patterns in two small segments of arm E and C (viz. 11A to 12D on arm E and 1A to 3D on arm C) and the chromocentre region in some detail. A detailed cytological map of these 2 segments, with reference to ^{3}H-thymidine labelling patterns, is shown in Fig. 2. For the sake of convenience, we have illustrated 10 different types of ^{3}H-thymidine autoradiographic labelling patterns of these two chromosome segments and the chromocentre in Fig. 3. In this figure, the analysed segments of the chromosome arms E and C and the chromocentre region belonging to a nucleus with a given type of labelling pattern, have been presented in composite sets. These composite sets have been arranged to show the proposed temporal sequence of the ^{3}H-thymidine labelling patterns in polytene chromosomes of *D. kikkawai* from initiation to termination of a given replication cycle. Table 2 gives an interpretation of the autoradiographic labelling of different sites (as detailed in Fig. 2) on these 2 chromosome segments in the 10 labelling patterns of Fig. 3. It must be stated that the 10 labelling patterns shown in Fig. 3, are only to show some steps during the replication cycle; obviously, there would be some intermediary labelling patterns occurring in the interval delimited by any two consecutive patterns chosen for illustrations here. This sequential arrangement of different labelling patterns is in keeping with the concept of uninterrupted synthesis of DNA at a given replication site[12], since no "exceptional patterns"[13,11] have been generated in these ordered arrays (Table 2). As there is considerable evidence for relating the 1D type of labelling patterns in polytene nuclei of *Drosophila* to the terminal phase of replication cycle[3,10,15] we suggest that in *D. kikkawai*, the LIB patterns represent the initial stages of a replication cycle. Thus the proposed temporal sequence of different labelling patterns from initiation to termination in *D. kikkawai* polytene nuclei would be as follows: LIB → MIB → HIB → 2C → 3C → 3D → 2D → 1D.

In LIB type of labelled nuclei, very few sites are labelled. The nucleus from which the two chromosome segments are illustrated in Fig. 3a is very interesting. In this particular nucleus only the E-11E 1–2 and E–12C 2 sites are seen to be labelled. In a few other LIB type of nuclei, an example of which is shown in Fig. 3b, the sites E-11E 1–2, E–12C 1–2 are labelled and in addition, C–3C 2 also shows a low labelling. Thus if, as suggested above, the LIB patterns represent the initial stages of a replication cycle, it would appear that in a polytene nucleus of *D. kikkawai* the replication cycle is initiated at E–11E 1-2 and E–12C 1 sites, closely followed by C–3C puff site.

As can be seen from Fig. 3 and Table 2, in MIB and HIB labelled nuclei, more of puff and interband regions become progressively labelled. In HIB labelling patterns, the ^{3}H-thymidine incorporation

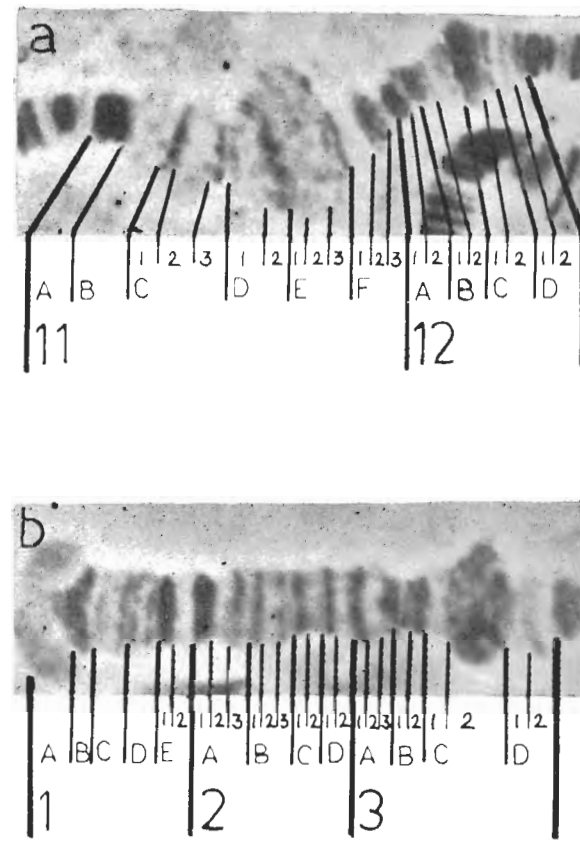

Fig. 2 — Detailed maps of the chromosome segments, 11A to 12 D of arm E (a) and 1A to 3D of arm C (b) shwoing the subdivisions identified with reference to sites of replication, × 2520

in the puffs E–11E and C–3C is striking since in almost all such nuclei, only these 2 puff sites are seen to be very heavily labelled with ^{3}H-thymidine; other puffs (e.g. 11B, 11C, 11D puffs on arm E and 2B on arm C) show a moderate level of incorporation. The E–12C 1–2 sites also show a fairly heavy labelling in HIB patterns.

Continuously and discontinuously labelled polytene nuclei in *D. kikkawai* show the usual features of 2C, 3C, 3D, 2D and 1D type of labelling noted in other species (Figs. 3f–j) but there are a few notable differences also. Interestingly the puff regions in 11B and 11C on arm E continue to have a low level of ^{3}H-thymidine incorporation in 3D and 2D type of nuclei. The E–12C region is low labelled in 2D type of nuclei. In 1D type of nuclei, all interbands and puffs (except E–11E and C–3C regions) remain unlabelled while dark bands show heavy ^{3}H-thymidine incorporation. The E-12C region, is unlabelled in 1D type nuclei. Significantly, in 1D type nuclei, while all other puff regions are unlabelled, E–11E and C–3C puff sites appear labelled. In E–11E puff, however, in some 1D type nucei, the ^{3}H-thymidine incorporation is restricted to the proximal half of the puff (i.e. sites E–11E 2–3, see Fig. 3j, and Table 2, column j) while the distal region (E–11E 1) is unlabelled. In some other nuclei classified as 1D type, the E–11E puff was

seen to be more extensively labelled than in the example in Fig. 3j, which shows a late 1D pattern. In the C-3C puff region also, in late 1D patterns as in Fig. 3j, the labelling appears to be restricted to the proximal region of the puff (C–3C 2 site).

Discussion

The ^{3}H-thymidine labelling patterns seen in larval salivary glands of *D. kikkawai* present several interesting features with respect to the temporal order and other aspects of polytenic replication cycles. The occurrence of many different types of interband labelling patterns, which can be arranged in ordered arrays (Fig. 3) leading to the continuous labelling patterns, reaffirms the belief[1,3,6] that the polytenic replication cycle in third instar larval salivary glands of *Drosophila* is inititated discontinuously at disperse regions. The interband type of labelling patterns have been seen in several species of *Drosophila* : in *D. hydei*[16], *D. melanogaster*[1,17], *D. pseudoobscura*[3,6], *D. athabasca* and *D. azteca*[5] and in *D. bipectinata* (Lakhotia, unpublished). In most of these studies, so many different types of interband patterns have not been described and also the interband patterns have been seen relatively less frequently; this paucity of interband patterns in other species of *Drosophila* has been suggested to be due to a shorter duration of the interband labelling phase in the S-period[3]. The present observations in *D. kikkawai*, however, show that in this species, the interband patterns are very frequent, the total frequency of different kinds of interband labelling patterns is slightly more than 25% of all nuclei. Furthermore, in certain preparations as many as 50% or more of the nuclei show interband labelling patterns. The high frequency of interband patterns in certain larvae suggests that in *D. kikkawai* the polytenic replication cycle in late third instar larvae may be initiated synchronously in a greater number of nuclei than in other species. The average frequency of different types of labelling patterns pooled for 31 different larvae of various ages can also be utilized for roughly estimating the duration of different labelling patterns in a replication cycle. From the pooled frequency data it appears that the LIB stage (specific labelling of E–11E, E–12C, C–3C and a few other sites) is very brief but the MIB and the HIB stages are more prolonged. The phase of continuous labelling (2C and 3C patterns) also appears to account for a major part of the S-period. Significantly, in *D. kikkawai*, the discontinuous patterns, particularly the 1D type are seen in very few nuclei and this may again reflect a shorter duration of these phases.

As already suggested in observations, the LIB patterns in *D. kikkawai* appear to represent the initial stages of a polytenic replication cycle. This is also supported by the labelling patterns of the chromocentre regions since as discussed in detail by Chatterjee and Mukherjee[3], the heterochromatic chromocentre region in polytene nuclei, as in other cell types of *Drosophila*[18,19] initiates its replication later than many other chromosomal regions and continues to replicate till the very late 'S'. Accordingly, in the present study, we have noted that the LIB type nuclei show almost no incorporation of ^{3}H-thymidine in the chomocentre while the MIB and HIB type labelled nuclei have a progressively greater labelling of chromocentre. Thus the ^{3}H-thymidine labelling patterns of the chromocentre support the temporal sequence of different labelling patterns suggested earlier in observations.

These considerations lead us to suggest that a new replication cycle in salivary gland nucei of late third instar larvae of *D. kikkawai* is initiated at E–11E, E–12C and C–3C sites since these sites are specifically labelled in LIB patterns. The E–11E and C–3C regions are typical puff sites, as evidenced by their morphology and by our observations on ^{3}H-uridine incorporation at these sites (unpublished data) and, therefore, expected to be labe-

TABLE 2 — DISTRIBUTION OF ^{3}H-THYMIDINE LABELLING OVER DIFFERENT SITES IN CHROMOSOME SEGMENTS OF ARM E AND C ILLUSTRATED IN FIG. 3

Chromosome	Sites	Autoradiographic sets in Fig. 3									
		a	b	c	d	e	f	g	h	i	j
E	11A	—	—	—	—	—	·	+	·	·	±
	11B	—	—	—	·	·	·	·	·	·	—
	11C 1	—	—	—	+	+	·	·	·	·	—
	2	—	—	+	+	+	±	±	±	+	—
	3	—	—	+	+	+	·	·	·	·	—
	11D 1	—	—	+	+	±	·	·	·	±	—
	2	—	—	—	—	±	·	·	·	—	—
	11E 1	+	+	+	+	·	·	·	·	—	—
	2	+	+	+	+	·	·	·	·	·	·
	3	—	—	+	+	·	·	·	·	·	·
	11F 1	—	—	±	±	·	·	·	·	±	—
	2	—	—	—	—	±	·	·	·	·	—
	3	—	—	—	±	+	·	·	·	—	—
	12A 1	—	—	—	—	—	·	—	·	·	·
	2	—	—	—	+	+	·	·	±	±	—
	12B 1	—	—	—	—	±	·	·	·	·	—
	2	—	—	·	+	+	·	·	·	·	—
	12C 1	+	+	+	+	·	·	·	·	±	—
	2	—	+	+	+	·	·	·	·	—	—
	12D 1	—	—	±	+	·	·	·	·	—	—
	2	—	—	—	—	±	+	+	±	±	—
C	1A	—	—	—	+	+	+	+	+	—	—
	1B	—	—	—	—	·	·	·	·	·	·
	1C	—	—	·	·	·	·	·	±	—	—
	1D	—	—	—	—	±	·	·	·	—	—
	1E 1	—	—	—	—	·	·	·	·	·	·
	2	—	—	—	—	·	·	+	·	—	—
	2A 1	—	—	—	—	±	·	·	·	·	·
	2	—	—	—	·	·	·	·	·	—	—
	3	—	—	—	—	±	·	·	·	·	—
	2B 1	—	—	—	—	·	·	·	·	·	—
	2	—	—	—	·	·	·	·	±	—	—
	3	—	—	—	—	·	·	·	·	—	—
	2C 1	—	—	—	—	·	·	·	·	—	—
	2	—	—	—	—	·	·	·	·	—	—
	2D 1	—	—	—	—	·	·	·	+	—	—
	2	—	—	—	—	·	·	·	+	—	—
	3A 1	—	—	—	—	±	·	·	+	—	—
	2	—	—	—	—	·	·	·	—	—	—
	3	—	—	—	—	·	·	·	+	+	—
	3B 1	—	—	—	—	·	·	·	+	—	—
	2	—	—	—	—	±	·	·	+	+	—
	3C 1	—	—	·	·	·	·	·	±	—	—
	2	—	·	·	·	·	·	±	+	+	+
	3D 1	—	—	—	·	·	·	·	—	—	—
	2	—	—	—	·	·	±	·	·	—	—

— = unlabelled
+ = labelled
± = part of a site labelled

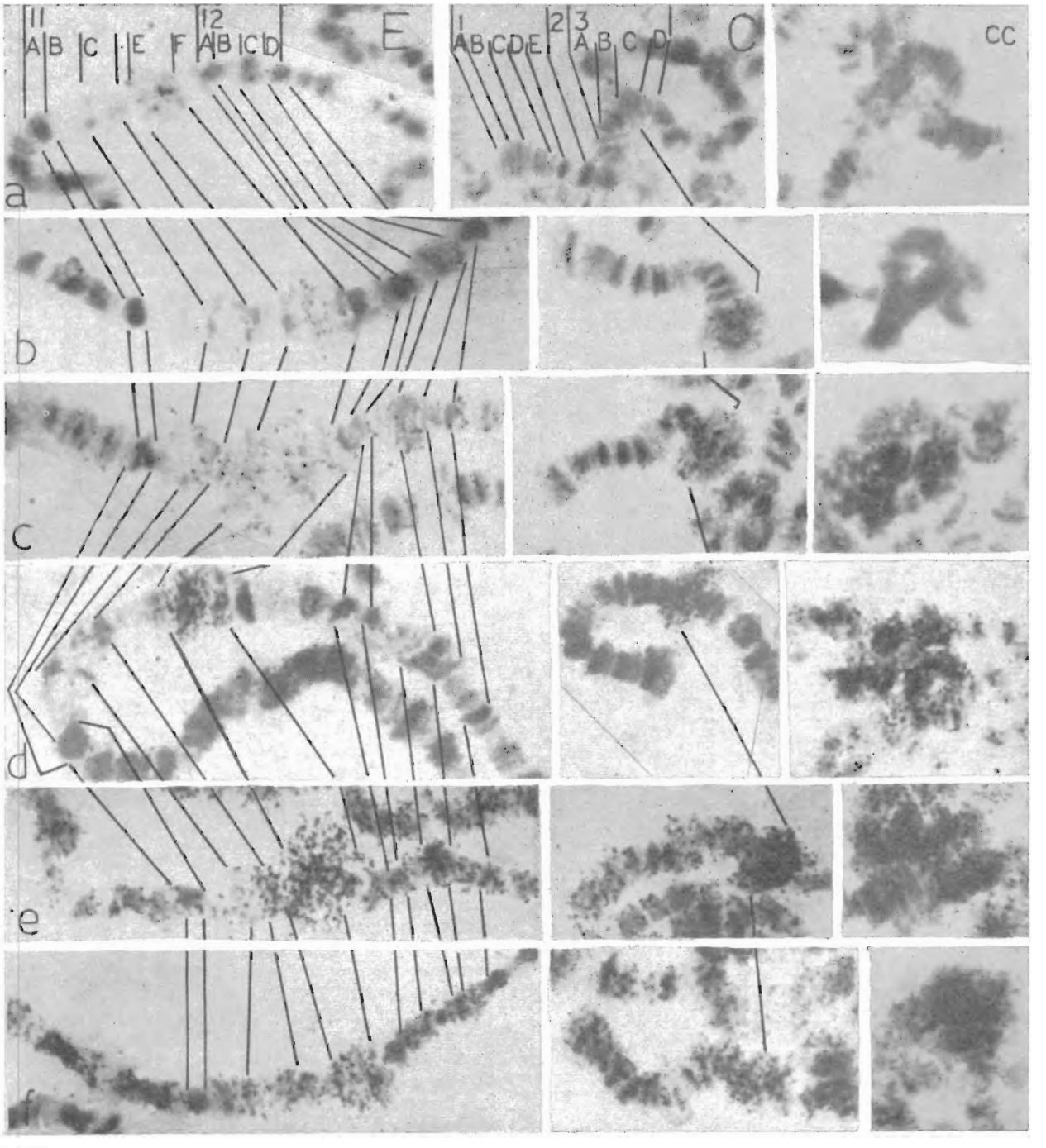

Fig. 3 — Representative examples of ^{3}H-thymidine labelling patterns on the chromosome segments of arms E (11A—12D) and C (1A—3D) and the chromocentre, arranged in composite sets **a** to j. In each set, the segment of arm E is placed on the left, the segment of arm C in the middle and the chromocentre (cc) region on the right side. Examples in the sets *a* and *b* correspond to LIB, c and d to MIB, *e* to HIB, f to 2 C, g to 3C, h to 3D, i to 2D and j to 1D types of autoradiographic labelling patterns. × 1600 [For details, see Table 2 and the Text]

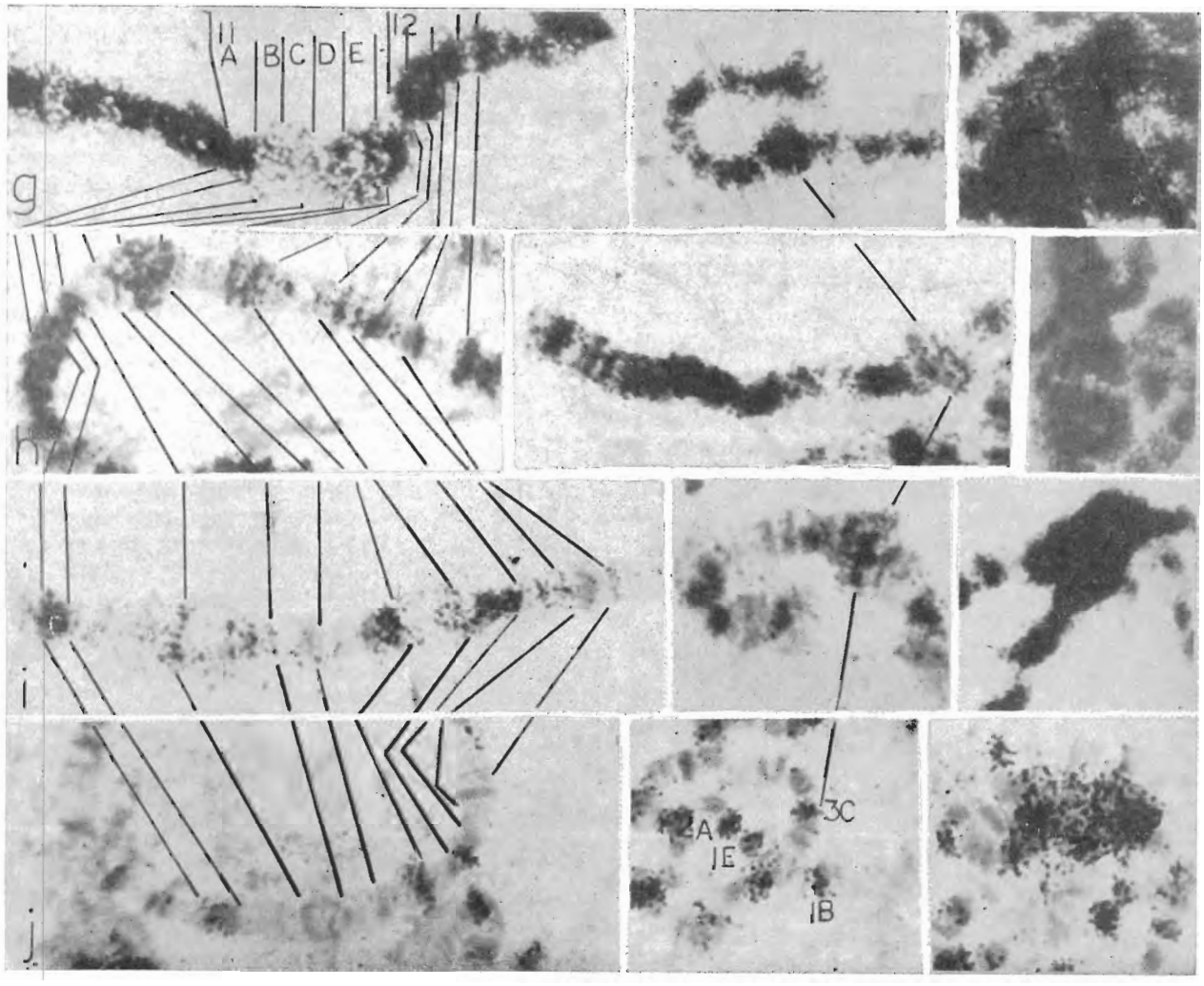

Fig. 3--(Continued from page 236)

lled with ^{3}H-thymidine in early part of the S phase. The labelling of E–12C region with ^{3}H-thymidine in LIB patterns is interesting since this region does not appear to be a puff; rather this region has a series of closely placed darkly stained bands which very often have a diameter greater than the adjoining regions (see Fig. 2). Thus it seems that in polytene nuclei of *D. kikkawai* a replication cycle is initiated by 2–3 replicating units and these include puff as well as banded regions. A similar initiation of replication cycle in polytene nuclei of *Drosophila* has not been reported so far in the literature. However, it is interesting to note here that in our studies on replication in polytene chromosomes of *D. nasuta* (unpublished data), we have recently observed a situation closely similar to that noted in *D. kikkawai*. Thus it may be suggested that in different species of *Drosophila*, the initiation of a polytenic replication cycle in salivary glands of late third instar larvae may be somewhat different: in general, the initiation occurs at the disperse chromosomes regions (interbands and puffs) but the synchrony with which the various puffs and interbands start replication may vary. In *D. kikkawai*, the initiation of different sites may appear to be greatly asynchronous so that one can observe the diverse kinds of interband patterns ranging from 1–2 sites labelled (LIB) to a labelling of nearly all interbands and puffs (HIB type).

The continued incorporation of ^{3}H-thymidine in E–11E and C–3C regions even in late 1D patterns is intriguing since in other *Drosophila* species, the early replicating puff and interband regions complete their replication cycle earlier than the bands[2,3]. In *D. kikkawai*, apparently, the 2 initiating sites (E–11E and C–3C) do not complete their cycle of replication earlier than the late replicating sites (bands and chromocentre). At the present state of knowledge we do not know if the E–11E or C–3C puff regions are composed of only one or more replicating units. In the latter case, it is possible to conceive that one or more of these units replicate in the early period and the other units do so in the latter part so that in our autoradiograms, the puff sites in question, show ^{3}H-thymidine incorporation in all labelling

patterns. That some such organization may be existing is indicated by a detailed examination of the site of ^{3}H-thymidine incorporation in E-11E puff in the different types of labelled nuclei. As shown in Fig. 2 and Table 2, we have identified at least 3 independent labelling sites in the E-11E region (E-11E 1, 2, 3), of which E-11E 1 and E-11E 2 (puffed faint bands) are labelled in the initial stages while E-11E 2 and E-11E 3 are labelled in late ID patterns. On the basis of present analysis, it appears, therefore, that the faint band at E-11E 2 site is labelled from the very initial to the terminal stage of replication cycle. In case of C-3C 2, we could not make a similar analysis since grains were always found scattered over this puff region. Nevertheless, the possibility remains that a site which starts replicating very early in the S, continues to do so till the very late S and this makes the temporal sequence of replication patterns in *D. kikkawai* somewhat different from the so far known examples in other species of *Drosophila* where the initial and terminal ^{3}H-thymidine labelling patterns of polytene chromsomes have been found to be complementary. The continued synthesis of DNA during the entire 'S' period at the two sites also raises the possibility of synthesis of "extra" DNA as is known for some sites in polytene chromosomes of *Chironomous*[20]. Further autoradiograhic labelling studies in combination with cytophotometric analysis may provide a satisfactory explanation to the curious ^{3}H-thymidine labelling patterns seen in the polytene chromosomes of *D. kikkawai*.

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