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 larva of D. kikkawai** has been studied by autoradiography of squashed preparations of salivary glands following a 10 min in *vitro pulse* of *3H*-thymidine. In addition to the usual continuous (all sites labeled) and discontinuous (combined bands and chromosomate units labeled) types of autoradiographic labelling patterns, a variety of intermittent (HR) types of labelling patterns have been seen with a relatively high frequency. The discrete reticular puff patches and interbands are clearly labelled in intermittent type of labelled patterns. Depending on the number and degree of labelling of these discrete regions, 3 major categories of HR patterns, all low (LRB), mild (MBH) and heavy (HRH) intermittent types have been recognized in *D. kikkawai*. The replication of 2 puff sites, *S* and *C* on arms *R* and *C*, respectively, of the reference photomap presented here, shows unusual features since during 3-4 hrs 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 (intermediate replicase regions) of a method is greatly accelerated and some of the initiating sites (e.g. puff *P* and C) 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 reports on *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 chromosome arms and bands (compact regions) start replicating later and totisomere 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 as at the end of the *S* and these 2 discontinuous types have been shown to be complementary to each other in several *Drosophila* species examined1. In the present study, we have examined *H*-thymidine labelling patterns in the *polytene chromosomes of Drosophila kikkawai*. The major point of interest in this species is the replication 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 *H*-thymidine labelling patterns in polytene chromosomes of *D. kikkawai* was presented earlier2.

Materials and Methods

A wild type strain of *D. kikkawai* (from Brasil) has been used in these studies. Larvae were grown in standard medium—agar medium at 20°C ± 1°C; salivary glands from late 3rd instar larva (black spade stage3) were dissected in *Drosophila* Ringer solution. The excised glands were pulse labelled for 10 min with *3H*-thymidine (250 μCi/ml; Sp. act. 10.4 C/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°C—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 acetocarmine 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 chromosome. The 5 long arms represent the X-chromosome (prolonged) and the left and right arms of the 2nd and 3rd pair of metacentric autosomes, respectively. In an earlier preliminary communication4 we presented a photomap of *D. kikkawai* in which we designated the 5 long arms as *Xl, 2L, 2R, 3L* and *3R,* 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 chromosome. 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 10 arms designated as A, B, C, D, and E represent the four pairs of the 2nd and 3rd pair of metacentric autosomes. Accordingly, we have prepared a revised photomontage of D. kikkawai polytenie chromosomes in Fig. 1. In this map, each of the long arm is divided into 30 divisions, numbered from 1 to 30, from tip to the centromere end. Each of the divisions is further subdivided into 4-6 subareas, numbered alphabetically.

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

**General patterns of labelling:** A total of 184 nuclei were examined from the labelling pattern of salivary glands of D. kikkawai. The nuclei have been taken at various intervals (over a period of 24 hr) after the initial uptake taken block so that the overall frequency of different types of labelling patterns during the lag phase, mitotic stages could be determined.

The general autoradiographic labelling pattern of polytene nuclei have been grouped into the following four categories: (a) labelling of only the interband regions; (b) labelling of only the intraband regions; (c) labelling of both the inter and intraband regions; and (d) labelling of the whole chromosome. These categories are given below, but some interesting deviations in these patterns are discussed later.

**Interband (1B type):** The nuclei of this type show a restricted labelling of interbands and puffs with none of the bands and chromosomes being either unlabelled or labelled. These patterns correspond to the 'residual discoid bands' (2B) pattern described by Chatterjea and Bardhugij (1963). The different interband patterns seem to be further subgrouped into the following 4 categories: (IB, mid (IB)) or heavy (HIB) interband labelling patterns; the pattern of the nucleus in such a stage; the pattern of the nucleus in such a stage; the pattern of the nucleus in such a stage; the pattern of the nucleus in such a stage. The IB pattern of a low labelling in its main division over 2-3 puffs and a few interbands. The IB class consists of a wide range of labelled nuclei with more puffs and interbands labelled with a low to moderate grain density. The MIB pattern, nearly all puffs and interbands are moderately labelled. Several bands are also labelled but many remain clearly unlabelled. In HIB, the chromosomes is almost unlabelled while in mid (IB) and low (IB) the chromosomes have been recognised following Rodman’s classification.

**Continuous type:** In these nuclei, the labelling is restricted mainly to the dark bands and the chromosome (compact regions); again following Rodman’s classification, heavy (3C), mid (2D) and low (1D) continuous patterns have been recognized.

On the basis of the above criteria for classifying the labelled nuclei, the 184 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 labelled and continuously labelled nuclei is very low in D. kikkawai during the stage of larva used for study. The continuous patterns (specifically the 3C type) are very common. However, most interesting is the relatively high frequency of the interband patterns, particularly the MB. 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 even in more than 50% of all polytene nuclei present in the preparation.

**Temporal sequence of 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 H-thymidine to polytene cells, relate to nuclei which are in different phase of a given polytene replication cycle (156, 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 the development of the general H-thymidine labelling patterns in D. kikkawai chromosomes, the patterns of H-thymidine incorporation in a few chromosomal sites (viz. 11E and 12C on arm E and 11D on arm F) is present at a very early stage of larva.
Fig. 1 — Presence of salivary gland polyploidy chromosomes of D. viridis.
3C on arm C) appeared to be rather unusual since
since three sites were seen to be labelled in almost
difficult case, except that E-12E was low labelled
neral, and therefore, to understand the temporal sequence of
different labelling patterns and to analyse the unusual replications
behaviour of the above mentioned sites, we have
examined the 3H-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 cyto-
logical map of these 2 segments, with reference to
3H-thymidine labelling patterns, is shown in Fig. 1.
For the sake of convenience, we have illustrated 10
different types of 3H-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 3H-thymidine labelling patterns in
polyten nuclei of D. kikkarai from initia-
tion to termination of a given replication cycle. Table 2
gives an integration 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 consec-
tutive patterns chosen for illustrations here. This sequen-
tial arrangement of different labelling patterns is
in keeping with the concept of uninterrupted synthe-
sis of DNA at a given replication site, since no
"exceptional patterns" have been generated in these
ordered arrays (Table 2). As there is abundant
scant evidence for relating the 1D type of labelling
patterns in polyten nuclei of Drosophila to the termi-
nal phase of replication cycle, we suggest that
in 1L1B of labelled nuclei, very few sites are
labelled. The nucleus from which the two chromosome
segments are illustrated in Fig. 3 is very interesting.
In this particular nucleus, only the E-11E 1-2 and
E-12C 1-2 sites are seen to be labelled. In a few other
1L1B type nuclei, an example of which is shown in
Fig. 1c, the sites E-11E 1-2, F-12C 1-2 are labelled and in
addition, C-3C 2 also shows a low labelling.
Thus, as suggested above, the 1L1B patterns represent
the initial stages of a replication cycle, it would appear
that in a polyten nucleus of D. kikkarai the replication cycle is initiated at E-11E 1-2 and E-12C 1 sites, closely followed by C-3C 2 puff site.
As can be seen from Fig. 2 and Table 2, in 11B
and 1H2 labelled nuclei, most of puff and interband regions become progressively labelled. In HH
labelling patterns, the 3H-thymidine incorporation

in the puff E-11E 2 and C-3C is striking since in almost all such nuclei, only these 2 puff sites are seen to be
very heavily labelled with 3H-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 HH
patterns. Continuously and discontinuously labelled polyten
nuclei in D. kikkarai show the usual features of 2C,
3C, 3D, 2D and 1D type of labelling noted in other
species (Figs. 3C) but there are a few notable differ-
ences also. Interestingly the puff regions in 11B and
11C on arm E, continue to have a low level of 3H-
thymidine incorporation in 2D and 2D type of nuclei.
The E-12C region is labelled in 2D type of nuclei.
In 1D type nuclei, all interbands and puffs (except E-11E 1C and C-3C 2) remain unlabelled while
dark bands show heavy 3H-thymidine incorpora-
tion. 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 nuclei, the 3H-thymidine incorporation is restricted to the proximal half of the puff (i.e. sites
E-11E 1-2, 3, see Fig. 3), 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. 3, which shows a late 1D pattern. In the C-3C puff region also, in late 1D patterns as in Fig. 3, the labelling appears to be restricted to the proximal region of the puff (C-3C 2 site).

**Discussion**

The 5H-thymidine labelling patterns seen in larval salivary glands of *D. kokkuvu* 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 that the polytenic replication cycle in third instar larval salivary glands of *Drosophila* is initiated discontinuously at discrete regions. The interband type of labelling patterns have been seen in several species of *Drosophila*; in *D. hydei* [8], *D. melanogaster* [9], *D. pseudoobscura* [8], *D. athabasca* and *D. azteca* and in *D. hippelata* (Lakhotia, unpublished). In most of these studies, 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 the short duration of the interband labelling phase in the S-period [9]. The present observations in *D. kokkuvu*, 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. kokkuvu* the polytenic replication cycle in late third instar larval may be initiated synchronously in a greater number of cells and may 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 MBH and the HBB stages are more prolonged. The phase of continuous labelling (3C and 3C patterns) also appears to account for a major part of the S-period. Significantly, in *D. kokkuvu*, the continuous 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. kokkuvu* appear to represent the initial stages of a polytenic replication cycle. This is also supported by the labelling patterns of the chromosomes seen in the polytene region in the larval nuclei, as in other cell types of *Drosophila* [9] initiates its replication later than many other chromosome 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 5H-thymidine in the chromosome while the MBH and HBB type labelled nuclei have a progressively greater labelling of chromosome. Thus the 5H-thymidine labelling patterns of the chromosome support the temporal sequence of different labelling patterns observed earlier in observations.

These considerations lead us to suggest that a new replication cycle in larval salivary gland nuclei of late third instar larva of *D. kokkuvu* is initiated at E-11E, E-12C and C-3C sites. As 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 the observations of 5H-thymidine incorporation at these sites (unpublished data) and, therefore, expected to be lab
Fig. 3 — Representative examples of "H-thymidine labelling patterns" on the chromosome segments of arms E (E1A—12D) and C (C1A—3D) and the centromeres, arranged in consecutive 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 centromere (c) region on the right side. Examples in the sets a to h correspond to 1LR, c and d to 1BR, e to 2LR, f to 2CR, g to 3LC, h to 3RC, i to 3RD and j to 1D types of autoradiographic labelling patterns. (For details, see Table 2 and the Text.)
lled with \(^3H\)-thymidine in early part of the S phase. The labelling of E-12C region with \(^3H\)-thymidine in L1B 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. neura* (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 polytene 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 puff) 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 (L1B) to a labelling of nearly all interbands and puff (L1H type).

The continued incorporation of \(^3H\)-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\(^{16}\). 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 \(^3H\)-thymidine incorporation in all labelling
patterns. Tho some such organization may be existing is indicated by a detailed examination of the site of β-thymidine incorporation in E-11E puff in the different types of labelled nucleic. As shown in Figs. 2 and Table 2, we have identified at least 3 independent labelling sites in the E-111 region (E-II, 1, 2, 3), of which E-II, 1 and E-II, 2 (puffed faint bands) are labelled in the initial stages while E-II, 2 and E-II, 3 arc labelled in late GD patterns. On the basis of present analysis, it appears, therefore, that the faint band at E-II, 3 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. akkermans somewhat different from the so far known examples in other species of Drosophila where the initial and terminal β-thymidine labelling patterns of polytene chromosomes have been found to be complementary. The combined 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 Chromo-

Further autoradiographic labelling studies in combination with cytophotometric analysis may provide a satisfactory explanation to the curious β-thymidine labelling patterns seen in the polytene chromosomes of D. akkermans.

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