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Replication in *Drosophila* Chromosomes: Part VIII—Temporal Order of Replication of Specific Sites in Polytene Chromosomes from 24°C & 10°C Reared Larvae of *D. melanogaster*

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Replication in *Drosophila* Chromosomes: Part VIII—Temporal Order of Replication of Specific Sites in Polytene Chromosomes from 24°C & 10°C Reared Larvae of *D. melanogaster*

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D. melanogaster larvae have been grown at low (10 C) and usual (24 C) temperatures to study the effect of growth temperature on pattern of replication of specific replicating sites in polytene nuclei. In an earlier study [Mishra and Lakhotia, *Chromosoma*, **85** (1982) 221], we suggested that the polytene S-period in cold-reared larvae is prolonged due to a greater asynchrony in the initiation and termination of different replicating units of a nucleus, while the number of replicating units and their rate of replication remain similar. In the present study, replication of different replicating units in specific segments of X and 3L in polytene nuclei from 24 and 10 C reared larvae has been examined in detail. This analysis provides further evidence for identity of replicating units at two growth temperatures and for a greater asynchrony in replication of different sites at low growth temperature. The results also suggest that the temporal order of initiation and termination of replication of different replicating units may vary in different nuclei within certain limits. Some aspects related to these are discussed.

Rearing of *Drosophila melanogaster* larvae at low temperatures (10° to 15°C) is known to result in 5 to 7-fold prolongation of the larval period along with some morphological alterations in salivary gland polytene chromosomes of late third instar larvae[1,2]. However, the maximum level of polyteny achieved by any nucleus is not correspondingly increased in cold-reared larvae and thus the polytene replication cycle is presumably longer at 10°C than at 24°C. In our earlier study[2], we suggested that the polytene S-period in salivary glands of cold-reared larvae is longer due to a considerable asynchrony in the initiation and termination of replication of different sites in a nucleus during a replication cycle, while the number of independently replicating units resolvable at the chromosomal level[3] and their rate of replication remain similar at the two (10 and 24 C) developmental temperatures. In the present study, we have examined the pattern of ^{3}H-thymidine incorporation in different regions of specific chromosome segments during the early or late polytene S-period in 10 and 24 C reared larvae. The results of this study provide further evidence for identity of the replicating units under the two rearing conditions and for a greater asynchrony in the initiation and termination of replication cycles of the different replicating units of a nucleus.

Materials and Methods

Wild-type (Oregon R) flies of *Drosophila melanogaster* raised at 24 ±1°C have been used. Eggs were collected from healthy flies in agar-cornmeal-brown sugar-yeast food at intervals of 1 hr, and allowed to hatch at 24°C. Immediately after hatching (about 22-24 hr), one group of larvae were transferred to 10° ±1°C and grown there (cold-reared group), while the other larvae were allowed to grow at 24° ±1°C (warm-reared group). Salivary glands from late third instar female larvae of the two temperature groups were dissected and pulse labelled with ^{3}H-thymidine (activity 250 μCi/ml, Sp. Act. 22.0 Ci/mM, Bombay) for 10 min at 24°C (for further details, see ref. 2). The labelled glands from cold- and warm-reared female larvae were fixed, stained and processed for autoradiography with Ilford K5 nuclear emulsion as usual. The exposure time was 9 days at 4-6°C.

Results

In order to analyse the replicative organization of specific sites in salivary gland polytene chromosomes under two rearing conditions, small segments of X-chromosome (1A-3F) and 3L (71A-75F) have been chosen for a detailed study. The autoradiographic labelling of different independently replicating sites ("replicating units" at chromosomal level, see ref. 4-7) in the selected X and 3L segments of nuclei showing the early (interband) and late (discontinuous) labelling patterns has been examined. A similar analysis has also been made for a segment of 2R (56A-60F) but the detailed results for the 2R segment are not presented here since they reflect the same general features as the X and 3L segments. In the following the labelling of different sites of the X and 3L segments is described in detail while only a few relevant aspects of labelling of 2R sites are noted.

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On the basis of autoradiographic labelling patterns in the analyzed polytene nuclei, 14 independently labelling sites in the X and 18 in the 3L segment could be delineated in cold- as well as warm-reared larvae. This delineation is based on the same criteria as adopted by earlier workers for identifying independently labelling sites in polytene chromosomes[4-7]. The cytological extents of the labelling sites identified on the X and 3L segments are presented in Fig. 1. The presence (a minimum of a cluster of 3 grains at a site) or absence of labelling at each of these sites of the X and 3L segments of well spread nuclei showing the mid-interband (early patterns) or discontinuous (late patterns, for details of the labelling patterns in these cold- and warm-reared larvae see ref. 2) type labelling have been recorded. These observed patterns have been arranged in matrices of ordered arrays[3,4,7] of early and late patterns, respectively, to determine the temporal sequence of initiation and termination of specific sites at the two developmental temperatures. The relative frequencies of labelling of each of these sites in early and late patterns have also been calculated to determine the rank order of initiation or termination of their replication.

Due to the very low number of nuclei showing the interband type labelling patterns in the warm-reared larvae[2], a detailed analysis of the initiation of replication of different sites in these two segments could be obtained only from cold-reared group. The pattern of labelling of the different sites in X and 3L segments observed in 35 mid-interband type female nuclei are presented in ordered arrays in Fig. 2a. On the basis of the labelling frequencies of different sites in these early patterns (Fig. 2 d&e) and the ordered arrays of the observed patterns (Fig. 2a) it appears that the 71DE puff region is the first (in the two segments analysed) to initiate replication followed by 75B, 74C-F on 3L, and 2B1-10 and 2B11-18 on the X segment in that order. However, it is striking to note that for none of the sites, the presence of labelling remains uninterrupted when the combined patterns of X and 3L segments are arranged in the ordered arrays (Fig. 2a). A few examples of the variable labelling of these early labelling puff sites in the X and 3L segments are presented in Fig. 3. An examination of the ordered arrays of patterns in Fig. 2a shows that the other disperse sites in the X and 3L segments are initiated to replicate at later stages but the sequence of their initiation in different nuclei also appears to be variable since the labelling of these sites cannot be arranged in uninterrupted sequence in the ordered arrays (see Discussion).

The observed patterns of labelling of different sites in different discontinuous type labelled polytene nuclei from warm- and cold-reared female larvae have also

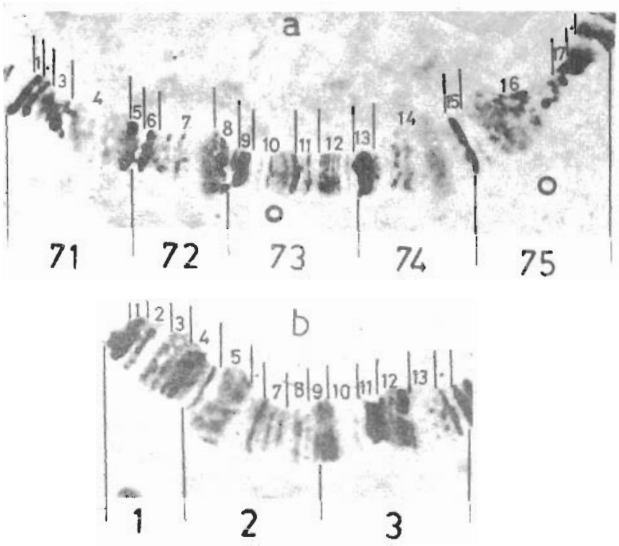

Fig. 1—Cytology of the segment of 3L (a) and X (b) of *D. melanogaster* analysed for ^{3}H-thymidine labelling of specific sites. The cytological divisions on standard polytene chromosome maps are indicated below the chromosome segments. The identity of each of the 18 "labelling sites" on 3L and 14 on X segment is indicated in the upper part of each figure. Magnification, X 1500

been arranged in matrices of ordered arrays to represent the possible sequence of termination of replication of the labelling sites (Fig. 2b-c). The ordered arrays as well as the labelling frequencies of different sites (Fig. 2d&e) reveal that the independently labelling sites on the X and 3L segments in 10° and 24° C reared larvae are cytologically identical. However, the frequencies of labelling of some 3L sites in the discontinuous type labelled nuclei show differences between 10° and 24° C reared larvae: e.g. sites 1, 6 and 9 on 3L show somewhat lower labelling frequency at 10° C than at 24° C, while the sites 4, 7, 14 and 16 (all puff regions) on the 3L segment show an increase in 10° C reared larvae. Other than these the labelling frequencies (in discontinuous type nuclei) of different sites on X and 3L segments are similar in the two temperature groups. As in the case of IB patterns noted above, in the ordered arrays of discontinuous labelling patterns also, the labelling of a given site does not remain uninterrupted. It is interesting that the so-called "exceptional patterns"[5] generated due to these interruptions in the presence or absence of labelling at a given site in the ordered arrays are more frequent in the cold-reared larvae (Fig. 2b and c). An estimate of the frequency of these "exceptional patterns" in the ordered arrays of discontinuously labelled X and 3L segments has been obtained by applying the following formula:

$$E_{LI} = \frac{\text{total exceptional labelling in the array}}{32 \times \text{No. of nuclei in the array}} \times 100$$

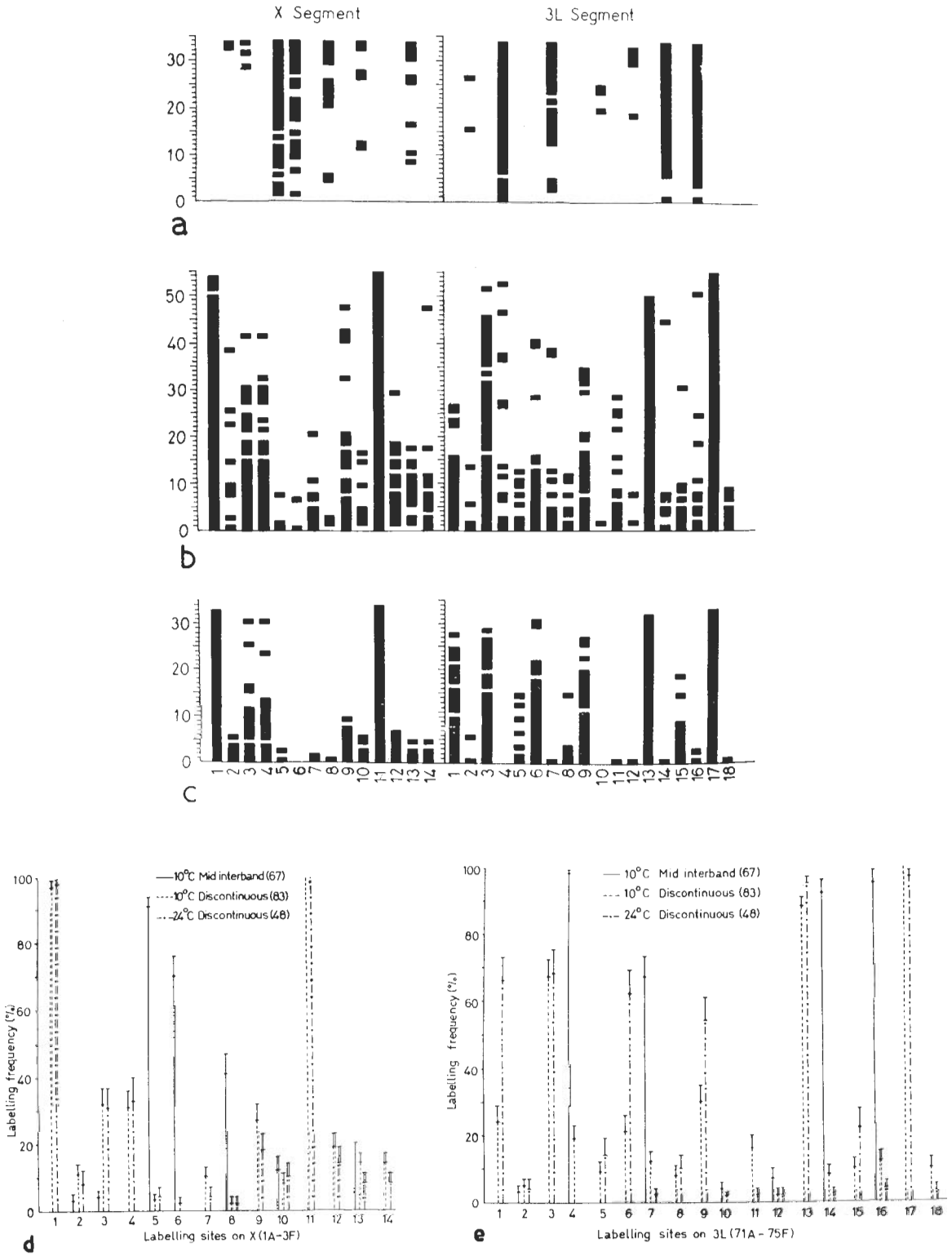

Fig. 2—a-c Ordered arrays of ^{3}H-thymidine labelling patterns of X and 3L segments from polytene nuclei of female larvae. (a) 10°C reared MIB type nuclei; (b) 10°C reared discontinuous type nuclei; (c) 24°C reared discontinuous type nuclei. Black bar indicates the presence of label at a site (abscissa). The labelling pattern of a nucleus (ordinate) is represented along one horizontal line. Some patterns were observed in more than one nucleus, but each pattern is shown only once in the ordered arrays. d-e ^{3}H-thymidine labelling frequencies of different sites on X (d) and 3L (e) segments in mid-interband and discontinuous type labelled nuclei.

(where, 32 is the total number of labelling sites, 14 on the X and 18 on the 3L segment, respectively). The E_{LI} (Exceptional Labelling Index) for the arrays of D type labelled nuclei at 24° and 10°C are 2.6% and 5.1%, respectively. Examples of such "exceptional patterns" seen on the 3L segment in cold-reared female larvae are presented in Fig. 4. The 3L segment in Fig. 4a shows labelling of all the heavier band regions and also the four puff regions; in Fig. 4b, only the dense band regions in the labelling site nos. 1, 3, 5, 6, 9, 13, 17 and 18 are labelled; at a later stage in Fig. 4c, the site nos. 5 and 6 become unlabelled, the site nos. 1, 3 and 9 are low labelled while the site nos. 13, 17 and 18 are fairly well labelled. In the examples in Figs. 4d and e, among the dense regions, only the site nos. 17 is distinctly labelled but the puff region in site 16 (75B, Fig. 4d) or in site 14 (74C-F, Fig. 4e) is also seen to be labelled. In the very late stage, in Fig. 4f, only the band region in the site 17 is labelled. In these examples, the labelling on any one of the two puff regions in Fig. 4d and 4e, is considered to be "exceptional" since in the ordered arrays in Fig. 4a-f, the presence of labelling at the puff 75B in *a* and *d* or at 74C-F in *a* and *e* is interrupted by no labelling in *b* and *c* or in *b*, *c* and *d*, respectively.

In our earlier study[2], we have shown that the independently labelling sites on 2R segment and their temporal order of initiation and termination is generally similar in the warm- and cold-reared larvae. As in the X and 3L segments, in the 2R segment also, the sites labelled in late S (1D type nuclei) are generally unlabelled in the interband type labelled nuclei seen in cold-reared larvae. The possible temporal sequence of

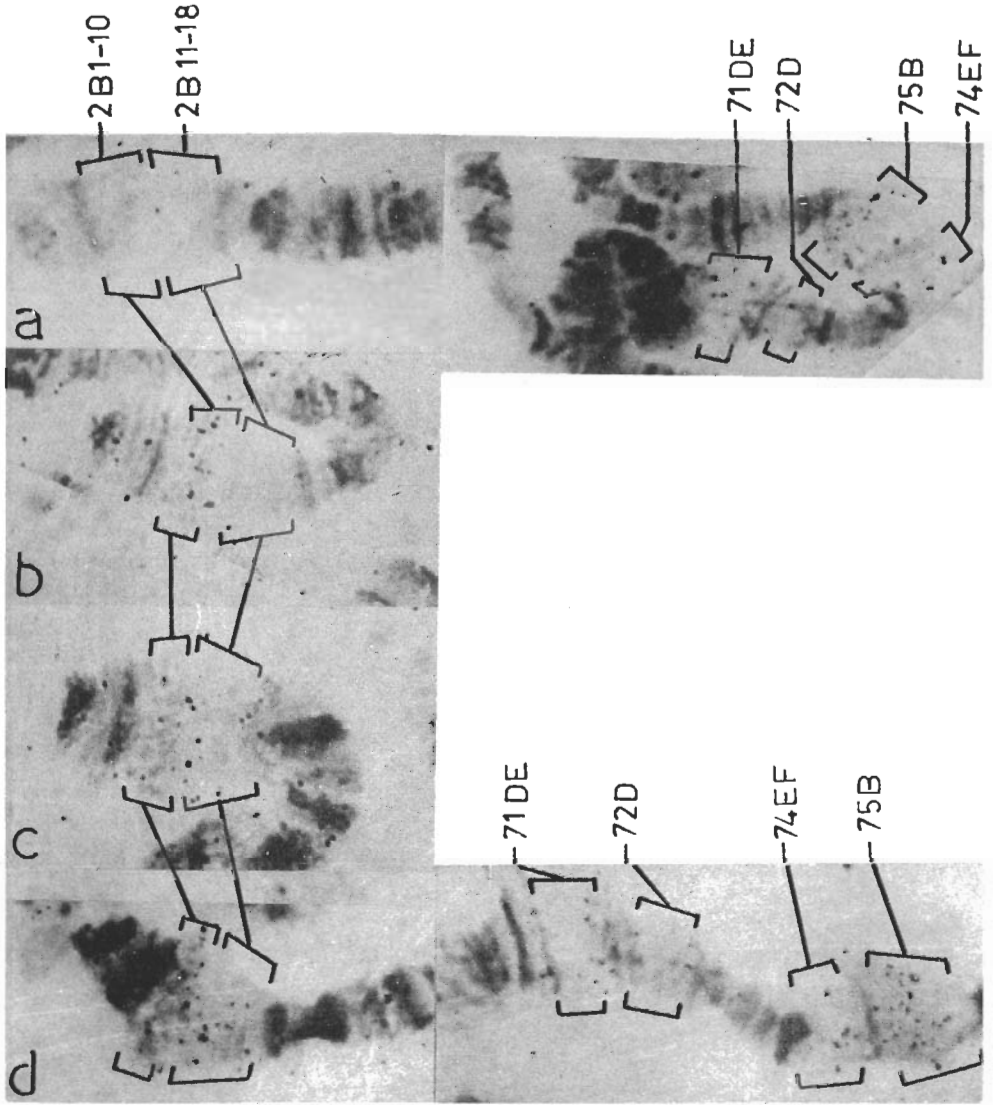

Fig. 3 Examples of variable ^{3}H-thymidine incorporation by the early labelling sites on 3L and X segments in different mid-interband type nuclei from cold-reared female larvae. In (a) the X segment is unlabelled while the puff sites 71DE, 74 C-F and 75B on 3L are labelled. In the other nuclei showing similar labelling on 3L (not shown), the site 2B1-10 (b) or 2B11-18 (c) is only labelled on X. At a still later stage (d), when 71DE, 72D, 74C-F and 75B are labelled on 3L, both the regions 2B1-10 and 2B11-18 on X are labelled. Magnification, X 1600

initiation of different sites on the 2R segment in cold-reared female larvae is shown in Figs. 5a-e, while the temporal sequence of termination of replication in the late labelling sites is shown in Fig. 5f-i. The puff region at 58 DE is the first to initiate DNA synthesis in this segment, followed by initiation in other disperse and later in other regions leading to continuous labelling stage. The pattern of initiation in the very late replicating 56F-57B region is interesting. Not only the dark bands in section 56F, 57A and 57B remain unlabelled or low labelled even till 2C stage (Fig. 5d, e), replication in the interband regions in this area is also initiated later than in other interbands. In the late 1D type nuclei, only the dense bands in the 56F-57B regions are seen to be labelled and of the 3 dense bands, that in 56F appears to be the last to complete its replication. "Exceptional" labelling patterns are seen in the ordered arrays of early as well as late labelling patterns of the 2R segment (data not presented), and as in the case of X and 3L segments, the incidense of "exceptional" labelling is higher in cold-reared larvae. Examples of "exceptional" presence or absence of labelling on some of the late replicating sites (like 56AB, 59D) are shown in Figs. 5f-i.

Discussion

The ordered arrays of labelling of specific replicating sites in the X and 3L chromosome segments confirm our earlier suggestion that the cytologically resolvable independently labelling sites ("replicating units" at the chromosomal level[3-7]) in salivary gland polytene chromosomes remain similar in spite of the differences in larval rearing conditions etc. On the basis of other studies on DNA fiber autoradiography of polytene chromosomes in our laboratory[8] it appears to us that in general the independently labelled sites identifiable at chromosomal level are reflection of the distribution of replication origin points. Thus, the identity of the "replicating units" at chromosomal level in the warm- and cold-reared larvae observed in this study would imply a similarity in the number and distribution of active replicons in polytene nuclei of warm- and cold-reared larvae.

The ordered arrays of labelling on specific segments of X and 3L also provide evidence for a greater asynchrony in the initiation and completion of

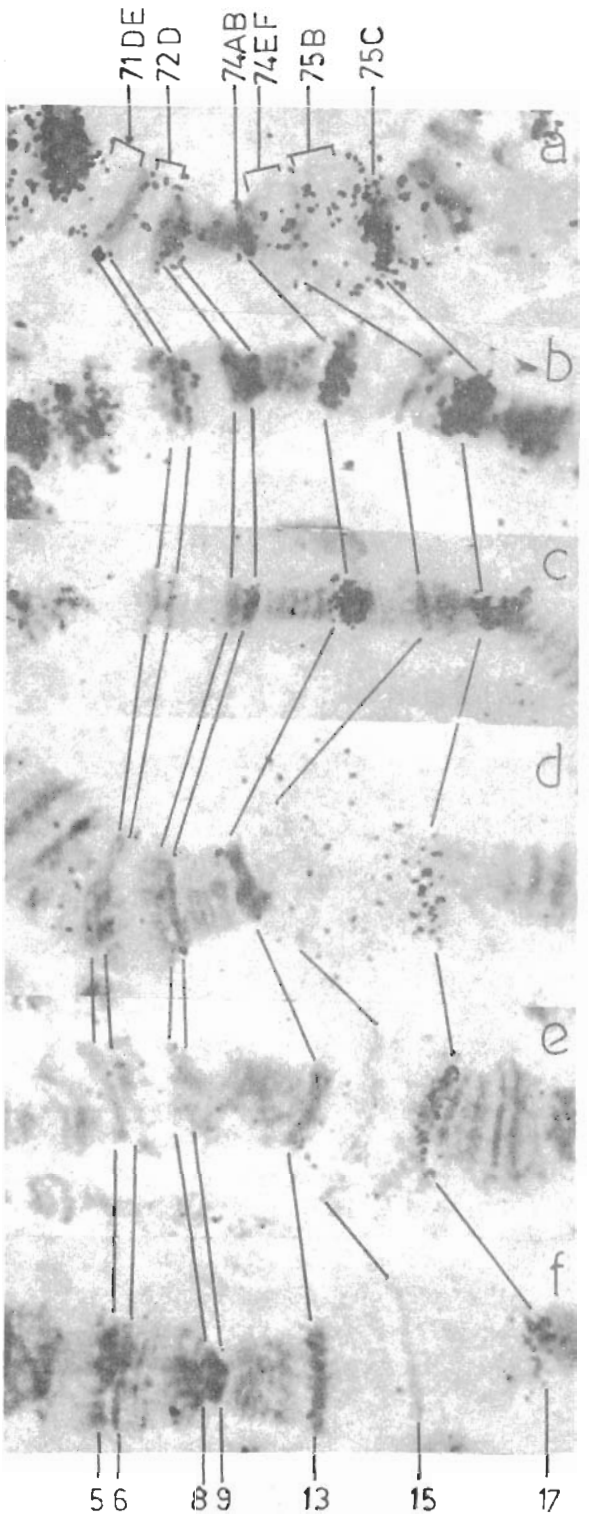

Fig. 4—Examples of variable ^{3}H-thymidine labelling of puff sites on the 3L segment in 1D type labelled nuclei from cold-reared female larvae. The different segments are arranged in the putative temporal sequence of termination from many sites labelled (a) to only 75C being labelled (f). Note the "exceptional" presence of labelling on the puff sites 75B (d) and 74C-F (e) at a very late stage of polytene S-period when only the late replicating sites like 75C (d) or 74AB and 75C (e) are labelled. Magnification X 1600

replication of the different replicating units in cold-reared larvae. As discussed in the earlier paper[2] the more frequent occurrence of interband labelling patterns in cold-reared larvae is indicative of a greater asynchrony in initiation of replication in the different disperse and dense chromosome regions. The large differences in the frequencies of labelling of the different disperse sites replicating in the early S-period (IB patterns) in cold-reared larvae is indicative of greater time interval between initiation of replication in the different regions. In warm-reared larvae, IB type labelling patterns are infrequent and moreover, the MIB type patterns are nearly absent[2] and, as such, there would not be such large differences between the labelling frequencies. Therefore, interval between the time of activation of origin points of different early labelling sites in warm-reared larvae would be smaller. Analysis of the ordered arrays of late labelling patterns reveals that many sites which are infrequently labelled in the D-type nuclei (late patterns) in warm-reared larvae are labelled more often in the D type nuclei in cold-reared larvae. It is significant that all sites which are completing their replication earlier in the warm-reared larvae do not show equal increase in their labelling frequency in D type nuclei in the cold-reared larvae. It may thus be suggested that since the initiation of replication of the different replicating units is more staggered at low temperatures, their termination is also correspondingly staggered. In this context, the relatively more frequent labelling of the puffs in sections 71, 72, 74 and 75 of 3L in the late patterns is somewhat unexpected, since these sites are also among the first to initiate a replication cycle. The increase in their labelling frequencies may imply that in cold-reared larvae the period of replication of these puff sites is longer than in warm-reared larvae. However, a few other possibilities may also be raised in this context for further studies. The occasional labelling of some of these puff sites in the 1D type nuclei from cold-reared larvae could also be due to a part of the DNA of the adjacent late replicating band (e.g. 74AB region near the 74C-F puff, and the 75C band near the 75B puff) being drawn into the puff structures at low rearing temperature. Hägele and Kalisch[9] have recently shown that even after puffing, a "late replicating" site remains late replicating. An alternative possibility could be that in some nuclei from cold-reared larvae a new replication cycle may be initiated before the previous ongoing cycle is completed, so that the early replicating puffs may occasionally appear labelled in 1D type nuclei. Chatterjee *et al.*[10] have shown that after inhibition of protein synthesis with puromycin, a new polytene replication cycle is initiated before the ongoing cycle is completed.

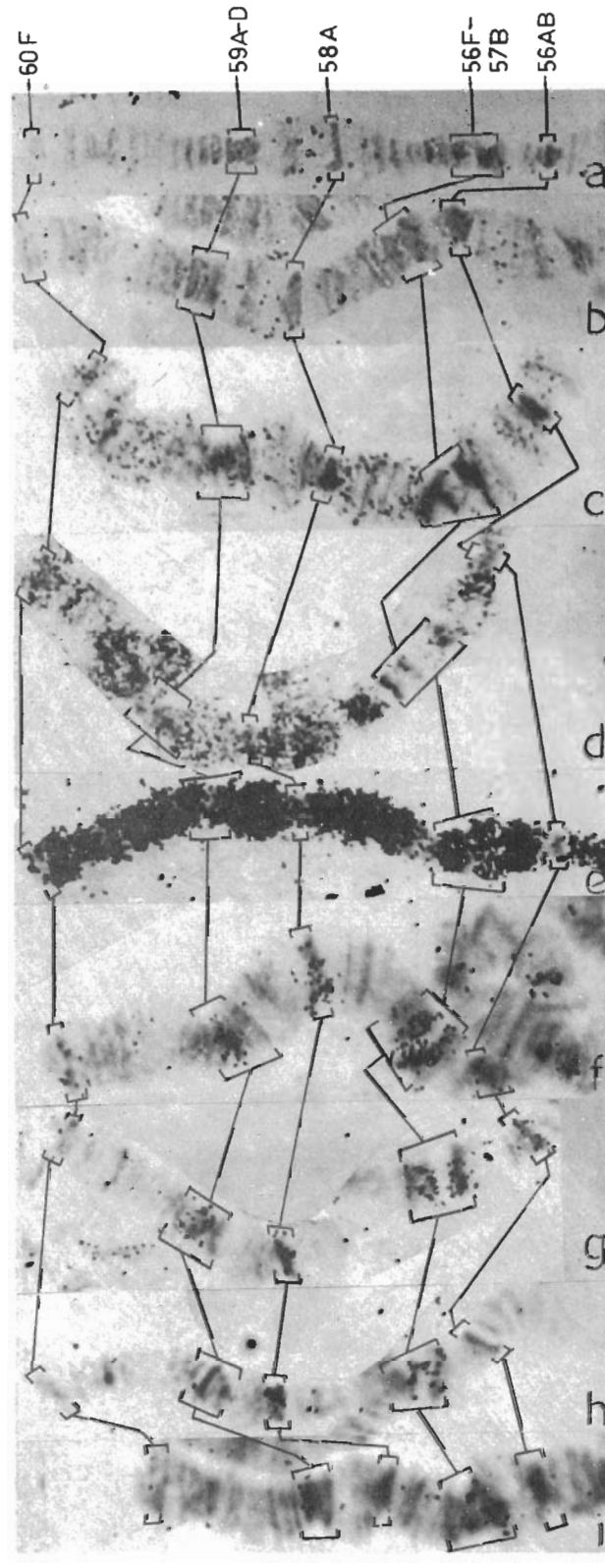

Fig. 5—Examples illustrating the possible temporal sequence of initiation and termination of replication of different sites on the 2R segment from salivary glands of cold-reared larvae pulsed with ^{3}H-thymidine for 10 min. Note the relatively late initiation of replication in 56F-57B region. For details see text. Magnification, X 1230

The decrease in the labelling frequencies of some late replicating sites (sites 1, 6 and 9 on 3L segment) in D type nuclei in the cold-reared group as compared to those in the warm-reared ones also appears to be a result of greater asynchrony in the initiation of their replication. Assuming that the duration of replication of a given site remains same at the two developmental conditions and that the very late replicating sites (e.g. 74AB and 75C) initiate their replication cycle later in the cold-reared group compared to that in the warm-reared group, the observed decrease in the labelling frequencies of some other sites in the former then does not appear to be an absolute decrease in its total period of replication, but only reflects a relative decrease in relation to the sites which are very late replicating and which thus invariably show a 100% labelling. The pattern of labelling of the very late replicating regions 56F-57B in the 1B and C type nuclei in cold-reared larvae (Fig. 5) is suggestive of a later initiation of such very late replicating sites in the cold-reared than in warm-reared larvae.

We have noted the so-called "exceptional" patterns in the ordered arrays of early as well as late labelling patterns in cold- and warm-reared larvae. We do not think that the "exceptional" patterns are generated due to technical artefacts as has been sometimes believed[5,7]. They appear to reflect an important aspect of polytene replication. One of the basic assumption made while arranging the observed labelling patterns of different nuclei in matrices of ordered arrays is that the temporal sequence of initiation and termination of replication of different sites is stringently regulated in all nuclei. The present observations, however, suggest that the temporal order of initiation and termination of replication of different sites is variable within certain limits. From the matrix of the early patterns in Fig. 2a, it is clear that even the temporal order of the initiation of replication of the very early replicating sites (viz. 71DE, 75B, 74C-F, 2B1-10 and 2B11-18 puffs) is variable in different nuclei, although several of these sites are always labelled in later M1B nuclei in which some other sites have also been initiated to replicate. Similarly, the order of termination of replication of the different late replicating sites is seen to vary in cold- as well as in warm-reared larvae; but again, if in a 1D nucleus a relatively less late replicating site (i.e. with a lower labelling frequency) is labelled, several, though not all, of the later replicating sites (with a higher labelling frequency) are also invariably labelled. From these it appears that in cold-reared as well as warm-reared larvae, there are "families" of replicating sites, so that between the different "families" the temporal order of initiation and termination is maintained, but, within a given "family" the temporal order of replication of different sites is variable. This organization appears to be a manifestation at chromosomal level of the probabilistic order of activation of one of the several potential origin points suggested at the DNA molecule level by Taylor[11]. It is possible that this probabilistic variability in activation of different origin points in polytene chromosomes of cold-reared larvae is enhanced due to their overall higher levels of polyteny.

Besides the above discussed assumption of a rigid temporal order of initiation and termination of replication of different sites, the other assumption in arranging the labelling patterns of different nuclei in matrices of ordered arrays is that the replication at a given site proceeds uninterrupted from initiation to termination and there is only one round of replication during one S-period[4]. Implicit in this is also the assumption that all the homologous origin points on lateral strands of a polytene chromosome are initiated to replicate synchronously. Both these assumptions may also not be valid. Recent results of Laird[12] have suggested that certain regions in a polytene chromosome are more replicated than others and this means that some sites may re-replicate during a given S-period. It has also been suggested that in chromosomes having higher overall polyteny levels, this aspect may be further exaggerated[12]. Results of DNA fiber autoradiographic studies of polytene chromosomes in our laboratory[8] (and other unpublished observations) have favoured Laird's concept of polytene chromosome organization, and have also suggested that the initiation of replication in homologous origin points on lateral strands of a polytene chromosome occurs asynchronously. These two features, viz. unequal polytenization of different euchromatin regions of a polytene chromosome and asynchrony in replication of the lateral strands would also contribute to the generation of "exceptional patterns" in the matrices of ordered arrays. It is likely that unequal replication of different regions and the lateral strand asynchrony will be more pronounced in nuclei with higher polyteny. Therefore, a greater incidence of "exceptional" presence or absence of labelling at a site, noted in the ordered arrays of labelled nuclei from cold-reared larvae, may be due to the cold-reared polytene nuclei having higher overall polyteny levels[1].

The present results thus reveal that although the cytological extents of the independently replicating units remain similar in polytene nuclei at the two developmental temperatures, the temporal order of replication of these units can be modulated independent of each other. This autonomy in programming their replication is probably the basis of the morphological and functional organization of polytene chromosomes[12].

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