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Environmental influences on puffing in the salivary gland chromosomes of *Drosophila melanogaster*

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SUMMARY

The sequence of development of salivary gland chromosome puffs in *Drosophila melanogaster* cultured on live yeast and RNA-deficient medium has been studied in late larval and prepupal stages. Differences in puffing pattern between individuals cultured in the two environments are almost entirely due to shifts in the relative timing of puff development at specific chromosome sites. Detailed studies on the right arm of the second chromosome indicate that, at certain sites, puffing activity spreads to adjacent lettered subdivisions of the chromosome. There are differences in the extent of lateral spreading of activity into adjacent regions in the two environments which may be due to clustering of functionally related genes.

1. INTRODUCTION

Beermann (1952) has shown that the occurrence of localized changes in the morphology of the polytene chromosomes of *Chironomus*, known as puffs and Balbiani Rings, is both age and tissue specific. He interpreted these changes as an expression of functional changes at the genetic level. The specificity of the pattern of puff formation to different tissues and developmental stages has similarly been demonstrated in *Drosophila melanogaster* (Becker, 1959, 1962; Ashburner, 1967). A puff involves localized swelling of the polytene chromosome associated with accumulation of acidic protein, and synthesis and storage of RNA within the puffed region (Pelling, 1964). Although there is some difference of opinion as to whether RNA synthesis is the only necessary prerequisite for puff formation (Clever, 1967; Berendes, 1968), the puffed regions of the chromosome are known to be active sites of synthesis of heterodisperse RNA molecules with DNA-like base ratios (Daneholt *et al.* 1969).

The puffing pattern in *D. melanogaster* tends to be concordant in replicate groups of larvae of the same strain under constant environmental conditions, but some strain differences in the occurrence of puffs have been reported (Ashburner, 1969).

Lychev (1965) finds that puffing incidence at specific sites is generally lower in inbred compared to massbred strains. The only known case in which larvae fail to develop the typical puffing pattern appropriate to the close of the third larval instar is in a lethal mutant (Becker, 1959).

Different approaches have been used to demonstrate that changes in the sequence of chromosome puffing may be induced by physiological factors. In *Chironomus* (Clever, 1861) and *Drosophila* (Becker, 1962; Berendes, 1967) hormones play a significant role in the induction of the normal pattern of activity at specific puff sites.

Becker (1959), studying the sequence of puffing in explant salivary glands of *D. melanogaster* larvae incubated in Ringer solution found that some of the gland cells developed a puff not normally observed to be active. Kroeger (1960) showed that changes in puffing pattern in salivary gland chromosomes of *D. buskii* can be induced by incubation of isolated nuclei in the preblastoderm cytoplasm of *D. melanogaster* embryos. Temperature shocks, and changes in the ionic concentration of the incubation solution each cause changes in puffing activity in the nuclei of explant salivary glands (Kroeger, 1964; Berendes, Breugel & Holt 1965; Ritossa, 1964). Whilst these studies show that puffing sequences are susceptible to modification, it is by no means clear that the induced changes form part of the normal repertoire of gene activity in the development of salivary gland cells.

The aim of the present investigation is to examine whether patterns of gene activity in larval salivary gland nuclei may be modified by environments which impose different physiological conditions for growth within the range of ecological competence of the species. *D. melanogaster* can complete development in the absence of dietary ribonucleic acid and is consequently capable of synthesizing the necessary purine and pyrimidine ribonucleotides. In the studies reported here the sequence of chromosome puffing was studied in larvae and pupae of a strain cultured on standard live yeast medium and on RNA-deficient synthetic medium. The results show that induced differences in puffing pattern in the two environments are almost entirely due to shifts in the relative timing of puff development at specific chromosome sites.

2. MATERIALS AND METHODS

The Edinburgh wild strain was used for all experiments to be described. Puffing patterns were studied in larvae and prepupae reared on a standard oatmeal medium supplemented with a thick suspension of live yeast during the second and third larval instars. Their puffing patterns were compared with those of aseptic larvae reared on a synthetic medium containing no RNA. The composition of the medium is given by Burnet & Sang (1963) and details of the experimental procedure by Sang (1956).

Eggs from 3-day-old females were collected for 1 h on agar plates at 25 °C and left at that temperature until hatching commenced. Newly hatched larvae were inoculated into fresh cultures and allowed to complete their development at 18 °C. Under both experimental conditions within culture differences in physiological age between individuals of identical chronological age were observed. To get a more precise measure of the age of a given larva, the morphology of the salivary glands was chosen as the criterion. During third instar the salivary glands undergo marked swelling. We have distinguished between 'not swollen', 'swollen' and 'very

swollen' salivary glands. Analysis of puffing patterns showed that these three stages roughly correspond to the patterns recorded by Ashburner (1967) for larvae aged up to 114 h, between 114 and 117 h, and 117–120 h respectively. For overall comparison between larvae and soft white prepupae the three larval stages cited above were grouped together.

Cytological observations were made using temporary lacto-aceto-orcein squash preparations. Active sites were scored on the basis of lettered subdivisions (Bridges, 1935). This arbitrary criterion was chosen for technical reasons, and the number of sites recorded does not necessarily reflect the number of puffs, since adjacent subdivisions may form one puff, or one subdivision may contain more than one puff (e.g. 60 E 1–5 and E 6–12). Where subdivisions contain more than one active site the frequency at that site which showed the highest activity was recorded. All sites believed to undergo puffing changes are recorded.

Not less than three of the large, distal nuclei were scored in each individual. A site was regarded as active if it showed diffuse structure. When unaccompanied by lateral swelling such a region was scored with puff-size 1, if some lateral swelling was also observable a score of 2 was given. In addition a score of 3 was assigned to those regions which showed diffuse structure and swelling to more than twice the width of the unpuffed chromosome diameter. A total of 370 individuals was examined.

3. RESULTS

Larvae cultured on RNA-deficient aseptic medium are smaller and develop at a slower rate than those kept on live yeast medium (Table 1). Their salivary gland chromosomes appear to be smaller and the banding appears to be more clearly defined, although the chromosomes as a whole are less deeply stained.

Table 1. *Duration of the larval period on live yeast and RNA-deficient media at* 18 °C

Medium	Time after laying when larvae cease to feed (days)	Time after laying when pupae appear (days)	Mean pupation time (h)
Live-yeast	8–11	8–12	234
RNA-deficient	15–19	15–22	432

Preliminary examination of the puffing pattern in chromosomes 2 and 3 was made on larvae and prepupae from live yeast cultures. There were 20 puffs recorded in 2L, 41 in 2R, 23 in 3L and 19 in 3R. RNA-deficient individuals showed an essentially similar pattern in that no large new puffs were observed, nor were any of the major puffs present in the series of individuals from live yeast cultures absent from the RNA-deficient larvae. However, there did appear to be differences in the time of appearance of many puffs, and the appearance of puffed regions differed with respect to activity at adjacent sites. Chromosome 2R was chosen for detailed study. Lychev (1965) has previously shown that the incidence of puffs at

specific sites in 2R is highly concordant in replicate groups of the same strain under constant environmental conditions.

Table 2 lists those sites previously recorded in 2R by Ashburner (1967), with two exceptions. Ashburner recorded 58DE, whereas we found a complex situation in this region, the highest frequency observed being at 58EF. Locus 60B has also been excluded, because in this region we found two different puffs with markedly different behaviour patterns, neither of which was comparable with the behaviour of 60B reported by Ashburner. For both of these regions it seemed meaningless to attempt a comparison between strains. For the remaining loci a comparison of

Table 2. *Comparison of puff sizes at some loci in chromosome 2R in larvae and prepupae*

(NS, S and VS denote larval salivary glands as not swollen, swollen and very swollen, respectively. Data from Ashburner (1967) have been converted as explained in the text.)

	Live-yeast				RNA-deficient				Ashburner			
	Larvae			Pre-	Larvae			Pre-	Larvae			Pre-
Site	NS	S	VS	pupae	NS	S	VS	pupae	1	2	3	pupae
42A	3	2	1·8	1·4	2	1·8	1	1	2	2	2	0
43E	1·9	2	2	1·3	1·5	1·5	2	2·1	2	2	2	2
44 (A)B*	0	0	0	1·9	0	0	1·5	2	0	2	2	0
46F	1·3	1·7	2	2·5	1	1	1·5	2	0	2	2	2·6
47A	1·8	1·9	2	2·5	1·8	2	2	2·4	2	2	3	2·3
47BC	1·8	1·6	2	2·0	1·5	2	2·2	1·8	1	1·3	2	2
48B	2	2	2	1·9	2	2	2	2	0	2	2	1
49F	0	2	2	2·5	0	1	1·7	2·1	0	0	0	2
50CD	2	2·5	2·3	2·1	2	2	2·1	2	2	2·3	3	2·3
52A	2	1·3	2	1·1	0	1·8	0	1	0	0	0	0
52C	1·7	2	2	1·7	0	1·5	0	1·5	0	0	0	0
55E	1·5	1·7	2·3	2	1·8	2	2·2	1·8	0	2	2	2
56D	2	2	2	2	2	1·8	2	2	0	0	0	2
58BC	2	1·6	2·2	1	1·9	2	2·5	0	2	2	0	0
59F	0	1·5	1·8	2	0	1	1·2	2·2	0	2	2	2

* Ashburner records 44AB, whereas only 44B was observed in our material.

puff sizes has been made by converting the data of Ashburner (1967) on the assumption that puff stages 2–4, 5–7, 8–10 and 11–13 respectively, correspond to the four developmental stages recorded by us, and using (+) = 1, + = 2 and + + = 3 to obtain puff size averages. Allowing for the approximation involved in this conversion, and bearing in mind that there were differences in culture medium and temperature between the two studies, a comparison of data in columns 1 and 3 of Table 2 suggests that there are strain differences in puff development at 49(E)F, 52A, 52C and 56D(E). Two of these loci (52A and 52C) also show differences between live yeast and RNA-deficient culture conditions, so that the differences between strains may depend only on the differences in environmental conditions employed by Ashburner and ourselves. There is a measure of agreement between

the figures for average puff size at other loci, which could be interpreted to mean that there is little variation in puff size. Our observations indicate, however, very considerable variation both in size and, to lesser extent, in the presence or absence of a puff between different cells within one gland and between different glands of individuals reared under identical conditions. This variation is convincingly demonstrated by Lychev (1965) and Lychev & Medvedev (1967).

In individuals from live yeast cultures some 65 of the 120 subdivisions of chromosome 2R exhibited some degree of puffing activity during the period of development in which detailed observations were made. Of these, 34 had not

Table 3. *Comparisons of the number of lettered subdivisions in chromosome 2R showing puffing activity in the larval and prepupal stages in yeast-cultured and RNA-deficient individuals*

		Live-yeast			RNA-deficient			
Comparison	Active sites	Larva	Prepupa	Both	Larva	Prepupa	Both	
Larval and prepupal stages	Specific to	4	23	38	8	11	48	
	Total	42	61	65	56	59	67	
Yeast-cultured and RNA-deficient individuals	Specific to	—	1	—	3	—	—	
	In common	—	—	—	—	—	—	64
	Total	—	—	—	—	—	—	68

previously been recorded. One previously described puff at 46A (Ashburner, 1967) was not found. Table 3 shows for each treatment condition the number of sites of puffing activity which are specific to the larval and prepupal stages and those common to both stages. Thus we find that of the 42 puffs present at different sites in 2R in larvae cultured on live yeast medium, four were specific to the larval stage and did not occur in prepupae. Of the 61 puffed sites present in prepupae, 23 were specific to the prepupal stage and did not occur in larvae. Thirty-eight sites showed puffing activity in both the larval and prepupal stages. Comparison with the corresponding figures for larvae cultured on RNA-deficient media shows that differences in puffing pattern do not primarily involve activity at different chromosomal sites, but activity of the same sites at different times in development. Thirteen sites become active in the larval stage on RNA-defficient medium but not until the prepupal stage on live yeast medium, so that there is a striking degree of similarity between the larval puffing pattern of RNA-deficient individuals and the prepupal pattern of yeast-cultured individuals.

The only puff apparently specific to yeast-cultured individuals was at 48F with a puff size score of 1. It was observed in relatively few larvae. Three puffs (50E, 54A and 55D) were recorded which were specific to larvae cultured on RNA-deficient medium. They were observed only in association with adjacent regions of puffing activity. 50E was continuous with 50D in some individuals and with 50C and 50D in others. 54A was continuous with 54B. 55D was continous with regions 55C and 55E, raising the question of whether this puff appears be-

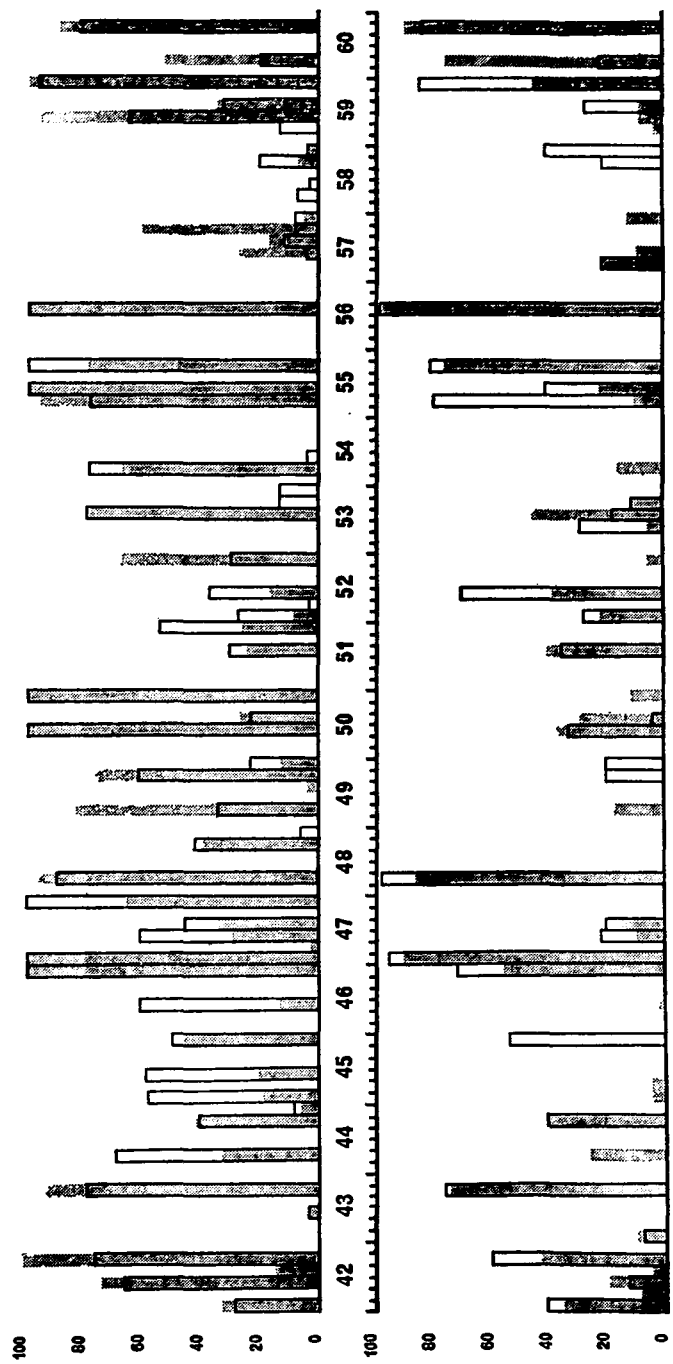

Fig. 1. Frequency of discrete puffs involving single-lettered subdivisions in chromosome 2R in larvae (lower distribution) and prepupae (upper distribution). Open columns, live yeast medium. Shaded columns, RNA-deficient medium.

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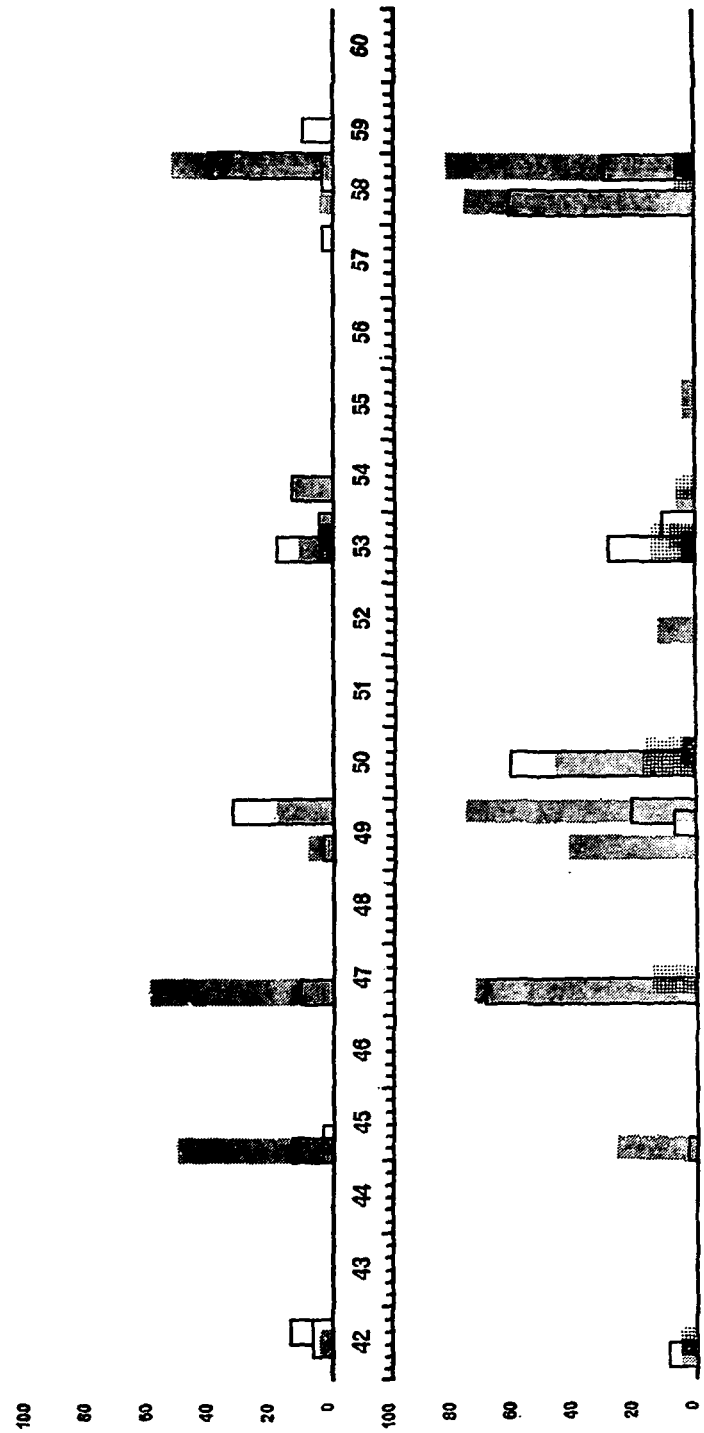

Fig. 2. Frequency of puffing activity involving two or more lettered subdivisions in chromosome 2R in larvae (lower distribution) and prepupae (upper distribution). Open columns, live yeast medium. Shaded, hatched and solid columns, RNA-deficient medium.

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cause of proximity to the active regions on either side, or because it constitutes a genuinely separate region of puffing activity. These observations are an indication of differences in lateral extent of activity of a region some part of which is common to both environments. Such an interpretation is not affected by the question of whether or not these are independent sites of puffing activity. Thus 42A–E, 45A–C, 47B–D, 52A–C, 53C–F, 54A–C, 58B–D and D–F are examples of regions where there are differences in the participation of adjacent sites in the activity of a site which is active under both environmental conditions (Figs. 1, 2).

Table 4 shows sites of activity classified according to whether puffs involve single, double- or triple-lettered subdivisions of the chromosomes. The data in the upper half of the table show that RNA-deficient larvae have more sites of activity of all three categories than the yeast-cultured controls. The last column of the lower half of the table indicates that although the majority of single active sites correspond in the two environments, the correspondence between the adjacent regions into which they appear to spread is less good. Figs. 1 and 2 show, for each recorded site, the frequency of activity observed. Some loci behave almost identically under both treatment conditions, while others show considerably frequency differences. Until further information is available as to the function of the loci concerned, only the most general interpretation is warranted. It may be that a large number of sites are concerned with functions in no way influenced by environmental conditions. The figures also show, within environments, variation in the behaviour of adjacent sites in relation to each other. They may appear to be discontinuous, suggesting the presence of simultaneous but distinct puffs, or give the appearance of one continuous puff. The observations indicate that such behaviour differs between environments. Thus 47CD, 49EF and 58EF in larvae, and 53EF and 58BC in prepupae, are all sites at which the yeast-cultured controls show two discontinuous puffs, whereas the two sites appear continuous in RNA-deficient individuals. Similar behaviour of such sites in different strains, under different environmental conditions and/or at different times during development, may account for the fact that, for example, Lychev (1965) and Lychev & Medvedev (1967) record a puff at 58E, Ashburner (1967) records a puff at 58DE, and our observations indicate the presence, at different developmental stages, of puffing at 58E, F, EF and DEF.

4. DISCUSSION

In the absence of dietary ribonucleic acid *D. melanogaster* larvae are capable of synthesizing ribonucleotides from simple precursors, but the efficiency of the constituent biosynthetic pathways is not adequate to support the normal growth rate. The larval period is greatly extended to give adult flies of small body size in which both cell size and cell number are reduced (Robertson, 1959, 1960). Under less severe nutritionally suboptimal conditions larvae are able to maintain normal body size by extending the period of development. To the extent that this device is no longer effective, RNA deficiency causes a decisive shift in the pattern of normal growth relations which may be reflected in changes in relative timing of puff de-

velopment at specific chromosomal sites demonstrated in the present data. There are two unknowns which hamper the interpretation of these results. On the one hand there is no reason to suppose that the salivary gland cells are active sites of ribonucleotide synthesis, a function which may well be sequestered in the fat body. Neither can we safely suppose that pathways of ribonucleotide biosynthesis are inducible, in the sense that larvae grown on live yeast medium, which contains

Table 4. *Comparison of the number of lettered subdivisions in chromosome 2R showing puffing activity in the larval and prepupal stages in yeast-cultured and RNA-deficient individuals, arranged according to the number of adjacent subdivisions involved*

		Live-yeast			RNA-deficient			
Comparison	Active sites	Larva	Prepupa	Both	Larva	Prepupa	Both	
Larval and prepupal stages	Singles							
	Specific to	5	24	30	7	15	35	
	Total	35	54	59	42	50	57	
	Doubles							
	Specific to	4	5	6	7	1	9	
	Total	10	11	15	16	10	17	
	Triples							
	Specific to	0	3	0	3	0	1	
	Total	0	3	3	4	1	4	
Yeast-cultured and RNA-deficient individuals	Singles							
	Specific to	—	1	5	1	2	1	
	In common	—	—	—	—	—	—	53
	Total	—	—	—	—	—	—	63
	Doubles							
	Specific to	—	1	3	5	—	1	
	In common	—	—	—	—	—	—	11
	Total	—	—	—	—	—	—	21
	Triples							
	Specific to	—	—	2	2	—	1	
	In common	—	—	—	—	—	—	1
	Total	—	—	—	—	—	—	6

optimum levels of dietary RNA, may also synthesize endogenous ribonucleotides from simple precursors. Defficiency of ribonucleotides, particularly adenylic acid would be expected to have a generally limiting effect on metabolism, whilst the demand for precursors for purine and pyrimidine synthesis is likely to alter the pattern of free amino acid utilization. Thus the consequences of RNA deficiency are probably extensive.

Thirteen puffing sites are active in 2R in the larval stage under conditions of RNA deficiency which appear only at the pupal stage in live yeast cultured individuals. Larvae raised under conditions of RNA deficiency evidently require the activity of some loci in the larval stage which individuals reared under normal conditions require only at a later stage of development, perhaps after they cease

to feed. The loci concerned might be involved, directly or indirectly, with RNA metabolism or with other factors influencing larval metabolism for which the two environments differ. It is conceivable that the RNA-deficient diet, whilst delaying development as a whole and puffing activity in many parts of the chromosomes, has not had this effect on other potential sites of activity. This would imply that we are distinguishing here between sites more directly related to development rate and those relatively independent of it. The latter may be more or less autonomous, or under some localized chromosomal control distinct from control which is more closely determined by metabolic requirements. The fact remains, however, that we are dealing with modulations of the time of appearance of puffing activity at specific sites rather than presence or absence. Provided a sufficiently broad time course is studied, the spectrum of puff activity is highly concordant between the two environments. The apparent differences in the occurrence of puffing activity at several sites in the *X*-chromosomes of larvae grown on normal and protein enriched culture media recorded by Lychev (1965) may well be due to similar adjustments in the timing of puff development since in these studies observations appear to have been confined to a single physiological age group.

Regional heterogeneity in puffing at a particular site related to differences in functional activity have been reported in *D. hydei* by Berendes (1965), who showed that secretion of gland substance by cells in the distal portion of the salivary gland is associated with the presence of a puff at 48B. Becker (1959) records that puffing at 15BC on the *X*-chromosome of *D. melanogaster* occurs only in the anterior portion of the gland. Also established by Lychev & Medvedev (1967), and confirmed in the present study, is that there are differences in the occurrence of puff activity at homologous chromosome sites between neighbouring cell nuclei within the same part of the salivary gland. Let us assume that the same sequence of puff activity is followed by all cells in the same region of the gland. If a prerequisite for activity at a given chromosome site is that the cell must reach some physiological threshold, then variations in adjacent cells in the time of reaching the threshold could, at any instant, lead to differences in the presence of a puff at that site, especially if the duration of puff development is short. There may well, of course, be gradients in the time at which cells in different parts of the gland reach the necessary threshold.

Movement of puff maxima along certain chromosome regions has been reported in *D. hydei* (Berendes, 1965), *Acricotopus* (Mechelke, 1961) and *Sarcophaga* (Whitten, 1969). The extension of puffs at particular chromosome sites into adjacent regions evident in our material could be a similar phenomenon. It may simply be due to independent development of puffing in overlapping time intervals at separate, functionally unrelated, loci situated close together on the chromosome. Until such sites have been investigated further, for example by tritiated uridine labelling, there is no evidence that this swelling of the chromosome is correlated with the production of mRNA. However, if one assumes that there is in fact local spreading of activity, several possibilities come to mind:

(i) Adjacent sites are not functionally related, and the spreading of puff activity

in the localized chromosome region could perhaps be attributed to some local and purely mechanical effect, such as uncoiling. This notion would seem to require that such a spreading effect should occur at any of the many observed sites of puffing activity whereas, as illustrated in Fig. 2, the sites at which it occurs tend to be clustered in particular regions of 2R.

(ii) Adjacent sites are related in the sense that there is functional linear redundancy of regions of the chromosome resulting from tandem interstitial duplication. Genetic evidence suggestive of such events is given by Bahn (1968) and Judd (1964), and cytological evidence by Keyl (1965*a*, *b*). Wider implications of the phenomenon are discussed by Callan (1967). This possibility would be credible in the present instance only if the degree of redundancy is extreme since, in contrast to the situation described by Keyl, we are dealing with adjacent lettered subdivisions of the chromosome which represent enormously long regions in molecular terms.

(iii) Adjacent sites are functionally related in the sense that their products participate in related metabolic processes. Clustering of functionally related genes is well illustrated by complex loci such as the bithorax pseudoallelic series (Lewis, 1964). If clustering is a fairly general phenomenon, some selective pressure must be responsible for maintaining such functional associations. Where genes are turned on or off by an efficient long-distance mechanism, there would appear to be no reason for any particular localization of genes, and one might therefore suppose that functionally related genes are spatially associated only when they are regulated by some essentially localized chromosomal control system.

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