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Replication in *Drosophila* chromosomes. IX. Stimulation of initiation of polytene replication cycles in vitro by juvenile hormone

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A greater proportion of polytene nuclei show [^{3}H]thymidine incorporation when third instar larval salivary glands of *Drosophila nasuta* are pulse-labelled after in vitro culture (3–24 h) in the presence of a juvenile hormone mimic, ZR 515. In glands chronically labelled with [^{3}H]thymidine in the presence of ZR 515, more nuclei are seen to have entered new polytene replication cycles. Similarly when salivary glands from larvae fed on 5-fluorodeoxyuridine to block polytene replication cycles at intersynthetic periods were cultured in vitro, new polytene replication cycles were initiated more quickly in the presence of ZR 515. These results suggest a stimulatory effect of juvenile hormone on new polytene replication cycles.

polytene replication juvenile hormone *Drosophila*

1. Introduction

The factors controlling the initiation of successive rounds of polytene replication in salivary glands of *Drosophila* larvae are not known (Berendes, 1973; Rudkin, 1972). A high rate of DNA synthesis has been observed during intermoult periods, while during periods of moulting or high ecdysone titer (Borst et al., 1974) the rate declines (Danieli and Rodino, 1967; Rodman, 1968). These and other (Sinha and Lakhotia, 1980) observations raise the possibility that insect hormones have some role in regulating polytenic replication in salivary gland nuclei. In the present study, we have examined the effects of juvenile hormone (JH), whose levels generally remain high during the intermoult periods (Riddiford and Truman, 1978), on polytene chromosome replication in larval salivary glands of *D. nasuta* cultured in vitro. Our results suggest that ZR 515 or methoprene, a potent JH mimic in *Drosophila* (Postlethwait, 1974; Richards, 1978), stimulates initiation of polytene replication cycles in vitro.

2. Material and methods

Eggs from a wild strain of *D. nasuta* (Varanasi) were collected at 30 min intervals and the larvae reared on standard food at 24° ± 0.5°C. A modified Poels' (1972) tissue culture medium has been used for in vitro culture of larval salivary glands (for details, see Sinha and Lakhotia, 1980).

2.1. In vitro culture of "unsynchronised" salivary glands

DNA synthesis cycles progress asynchronously in different nuclei of salivary glands from mid third instar larvae (Rudkin, 1972) and, therefore,

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in vitro cultures of these glands are referred to as "unsynchronised" cultures. Sister salivary glands from mid third instar larvae were separated and cultured for 3, 6, 12 or 24 h in the presence (treated) or absence (control) of ZR 515. The doses of ZR 515 were 3.0 μl ($\sim 9 \times 10^{-6}$ M), 0.03 μl and 0.0003 μl per ml of medium. For each experiment, 1 μl of ZR 515 (Zoecon Corporation, California) was first dissolved in 10 μl of 70% ethanol and this was freshly mixed with appropriate quantities of the culture medium by sonication or by shaking in a Vortex mixer; in the control cultures an equivalent amount of 70% ethanol was added. At the end of the culture period the control and treated salivary glands were pulse-labelled with [^{3}H]-thymidine (250 μCi/ml, spec. act. 12.5 Ci/mM, BARC, Trombay) in fresh medium for 20 min, fixed in 1 : 3 acetomethanol, squashed and processed for autoradiography (see below).

In another experiment, freshly excised sister salivary glands from mid third instar larvae were separated and chronically labelled with [^{3}H]-thymidine (2 μCi/ml, spec. act. 12.5 Ci/mM) in the absence or presence of ZR 515 (3.0 μl/ml) for a 24 h period. After the 24 h period, the glands were fixed, squashed and processed for autoradiography.

2.2. In vitro culture of salivary glands blocked in vivo with 5-fluorodeoxyuridine (5-FdUr)

In another experiment, 5-FdUr feeding has been used to block polytene replication cycles in vivo (see Achary et al., 1981). Since most of the replicating nuclei in these glands are blocked in early S phase (see Results), in vitro cultures initiated with glands blocked by 5-FdUr will be referred to as "synchronised" cultures.

The 5-FdUr feeding medium was prepared by mixing 200 μg 5-FdUr (Sigma) and 10 mg agar with 1 ml of the modified Poels' medium (without yeast extract); the mixture was heated to dissolve the agar. Mid third instar larvae (110 h post-oviposition) were fed on the 5-FdUr medium for 24 h.

In one set, salivary glands from the 5-FdUr-fed larvae were dissected out and immediately pulse-labelled (20 min) with [^{3}H]thymidine as above; the labelled sister salivary glands were fixed and squashed separately to ascertain intra- as well as intergland synchrony in replication after 5-FdUr feeding.

In a second set, sister salivary glands from 5-FdUr-fed larvae were separated and cultured in vitro in the absence or presence of ZR 515 (3.0 μl/ml) for 2, 6, 10, 20 or 30 h. After this period the glands were pulse-labelled with [^{3}H]thymidine as above.

All squash preparations were processed for autoradiography with Ilford L4 nuclear emulsion as described earlier (Lakhotia and Roy, 1979). Autoradiographic exposure for pulse-labelled preparations was 4 days, while that for chronically labelled glands was 6 days. For all the above experiments data were collected from duplicate or triplicate batches of three to four glands each, and for each experiment at least 250 nuclei were scored.

3. Results

A given polytene S period is presumed to progress in the following sequence in vivo as well as in vitro (Sinha and Lakhotia, 1980; Roy and Lakhotia, 1981). During the initial S period interband and puff regions replicate (interband-labelling patterns). This stage is followed by the continuous (C) labelling patterns during which all the chromosomal sites replicate. Finally, during the late S period, band regions are seen to replicate and, depending upon the intensity of labelling and the number of labelled bands, three types of discontinuous patterns (viz. 3D, 2D and 1D) are identifiable (for further details, see Sinha and Lakhotia, 1980; Roy and Lakhotia, 1981). In the present study, all these different kinds of labelling patterns have been seen in the control and ZR 515-treated glands pulse-labelled with [^{3}H]-thymidine. For the sake of brevity, however, the different interband and continuous labelling patterns have been grouped together as "early" and all discontinuous patterns as "late" patterns when comparing frequencies of the various autoradiographic patterns in the control and treated glands.

3.1. Labelling patterns in unsynchronised salivary glands cultured in presence of ZR 515

The frequencies of labelled nuclei as well as those of nuclei showing early and late patterns of [^{3}H]thymidine labelling in groups of sister control and treated glands are presented in Fig. 1A. The relative frequencies of replicating nuclei in the treated glands in comparison to those in sister control glands are shown in Fig. 1B. With all three doses of ZR 515, the treated glands at all time intervals (except 3 h samples) show higher frequencies of labelled nuclei compared to the sister control glands. In the untreated glands, the frequency of replicating nuclei gradually declines with time; in 24 h control samples of the different dose series only 5–30% replicating nuclei are seen (Fig. 1A). However, in the ZR 515-treated glands such a decline is not noted: in the different dose series, 50–80% nuclei continue to show autoradiographic labelling even after 24 h culture period (Fig. 1A). The higher frequencies of replicating nuclei in the treated glands are mainly due to an increase in the frequency of nuclei showing late patterns of [^{3}H]-thymidine labelling (Fig. 1A). It is significant, however, that after 24 h the frequency of early patterns is also higher in ZR 515-treated glands. Of the different doses tested, 3.0 μl/ml gave the maximum increase in the relative frequency of replicating nuclei in the 24 h sample. This dose has, therefore, been used for all subsequent experiments.

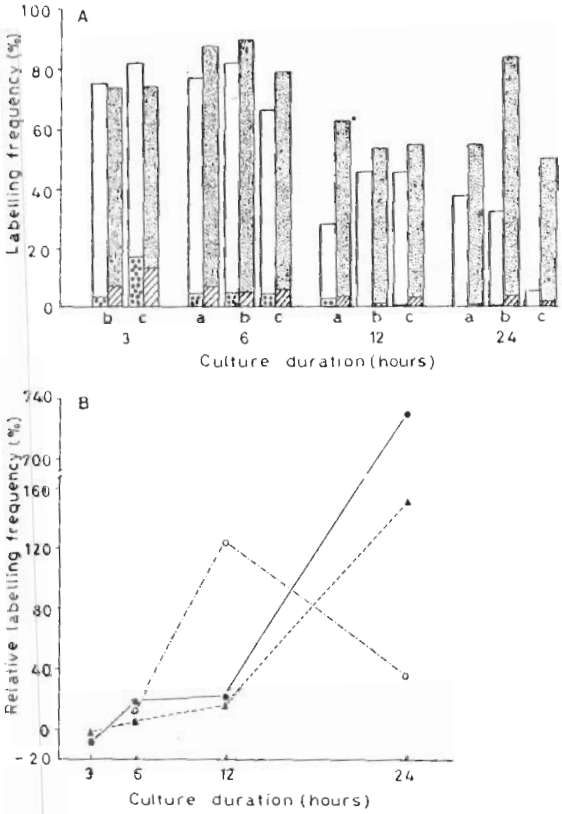

Fig. 1. A) [^{3}H]Thymidine-labelling frequencies in control and ZR 515-treated unsynchronised salivary glands at different time intervals of in vitro culture. a, b and c represent 0.0003 μl/ml, 0.03 μl/ml and 3.0 μl/ml respectively. In each case the left bar represents control and the right bar the treated samples. The height of the bar indicates the total labelling frequency, while the subdivisions show the frequencies of early (▨, control, ▩, treated) and late (□, control, ▣, treated) patterns, respectively. B) Relative frequencies of labelled nuclei in ZR 515-treated glands in comparison to those in sister control glands at different time intervals of culture. The relative labelling frequencies are derived by calculating the % difference (increase or decrease) between the total labelling frequencies in corresponding control (taken as 100%) and treated samples shown in A. ●, 3.0 μl/ml; ▲, 0.03 μl/ml; ○, 0.0003 μl/ml.

In autoradiographs of salivary glands labelled continuously for 24 h with [^{3}H]thymidine in vitro, some nuclei remain unlabelled (UN in Fig. 2). Among the labelled nuclei, some show the differ-

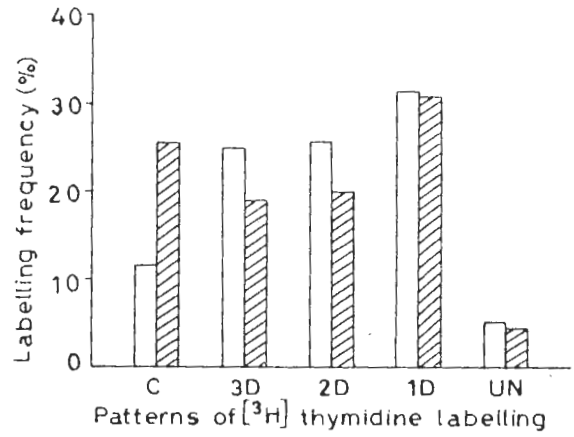

Fig. 2. Frequencies of continuous (C) and different discontinuous (3D, 2D and 1D) labelling patterns and of unlabelled (UN) nuclei in unsynchronised glands chronically labelled in vitro with [^{3}H]thymidine for 24 h in the presence (▨) or absence (□) of 3.0 μl/ml ZR 515.

TABLE I

Percentage of different [^{3}H]thymidine labelling patterns in sister salivary glands of four mid third instar larvae fed on 5-FdUr (200 μg/ml) medium for 24 h

Larva No.	Gland No. [a]	% Different patterns amongst the labelled nuclei			Total (%) labelled	No. of nuclei observed
		Interband	Continuous	Discontinuous		
1	a	73.96	3.13	22.92	80.00	120
	b	70.10	1.03	28.87	87.39	111
2	a	84.09	0.00	15.91	68.75	64
	b	88.46	5.77	5.77	85.25	61
3	a	96.30	0.00	3.71	34.62	78
	b	73.33	23.33	3.33	26.55	113
4	a	90.00	10.00	0.00	11.36	88
	b	92.31	7.69	0.00	12.75	102

[a] Gland Nos. a and b represent the two sister glands of a larva.

ent discontinuous (3D, 2D and 1D) types of labelling, while others show continuous labelling (C). No interband type-labelled nuclei are seen in these preparations. The frequencies of unlabelled and 1D type nuclei are similar in control and ZR 515-treated glands. However, the continuous patterns (C in Fig. 2) are nearly twice as frequent in the treated than in the sister control glands; the 3D and 2D patterns are correspondingly fewer (Fig. 2).

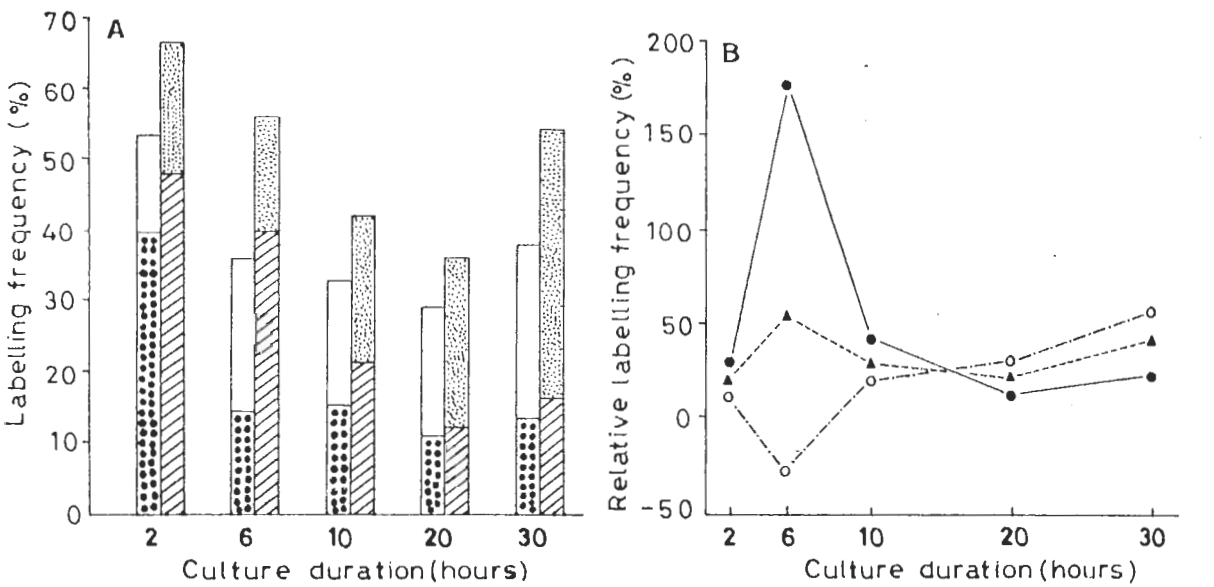

Fig. 3. A) [^{3}H]Thymidine-labelling frequencies in control and ZR 515 (3.0 μl/ml)-treated 5-FdUr-synchronised glands at different time intervals of in vitro culture. For explanation, see Fig. 1A. B) Relative frequencies of early (●) and late (○) patterns of [^{3}H]thymidine labelling and of total (▲) labelled nuclei at different time intervals in the ZR 515 (3.0 μl/ml)-treated glands in comparison to those in control glands. The relative frequencies have been calculated from data shown in B. For explanation, see Fig. 1B.

3.2. Effect of ZR 515 on new in vitro polytene replication cycles in salivary glands blocked in vivo with 5-FdUr

When salivary glands from 5-FdUr-fed larvae are released from the block by a brief pulse of [^{3}H]thymidine, in different pairs of glands a variable proportion of nuclei (10–90%, see Table I) incorporate the label. In all the glands, however, nearly all of the replicating nuclei show early patterns of labelling with 70–90% in the interband and 2–10% in the continuous labelling phase (Table I). Although the release in polytene S period in glands from different larvae occurs variably, the two sister glands of a larva show a closely comparable release, since the frequencies of different labelling patterns between the two sister glands of each 5-FdUr fed-larvae (Table I) are similar.

As in the unsynchronised glands, in the 5-FdUr-synchronised cultures also the frequency of labelled nuclei at different time intervals is higher in the ZR 515-treated than in the control glands (Fig. 3). Analysis of the relative labelling frequencies of early and late patterns in the treated glands (Fig. 3B) reveals that the increase in the frequency of the labelled nuclei in 2, 6 and 10 h samples is mainly due to a greater proportion of early patterns (Fig. 3B), whereas the late patterns contribute more to the increased frequency of labelled nuclei in the treated glands in 20 and 30 h samples.

4. Discussion

We have compared the frequencies of different labelling patterns in ZR 515-treated and control glands to ascertain the effects of the JH mimic on specific stages of polytene replication cycle. However, in view of the variability in the frequencies of replicating nuclei in different batches of control glands cultured in vitro for equally long periods, we have compared the labelling patterns only between sister salivary glands cultured in vitro in the presence or absence of ZR 515; it is known that between the two sister salivary glands, the polytene replication cycles in different nuclei progress synchronously (Roy and Lakhotia, 1977), and as the present study shows this synchrony is retained even after 5-FdUr feeding (Table I). The results, therefore, can be ascribed to treatment rather than to sample variability.

A given polytene replication cycle is presumed to traverse through the different early and late patterns of [^{3}H]thymidine labelling (see Results), while the successive rounds which progress asynchronously in different nuclei of a gland are separated by intersynthetic period of variable lengths (Rudkin, 1972; Achary et al., 1981). Indirect calculations show that the early patterns occupy a relatively shorter part of the polytene S period than the late patterns (Achary et al., 1981; Steinemann, 1981). During normal development, all nuclei in a salivary gland do not achieve similar levels of polyteny. There is a definite programme of endoreduplication cycles so that the posterior nuclei achieve higher polyteny whereas the polyteny level is decreasingly lower in more anterior nuclei (Rudkin, 1972). In view of this developmental programme, the observed increase in the frequency of labelled nuclei at different time intervals of ZR 515 treatment of unsynchronised glands may be due to one of the following reasons: i) ZR 515 treatment causes prolongation of the S period so that the replicating nuclei take a longer time to complete a given synthetic cycle and, therefore, upon pulse-labelling at any time, more nuclei appear labelled in the treated glands; ii) a greater proportion of nuclei in the ZR 515-treated glands enter a new replication cycle in vitro than in the sister control glands and, therefore, the frequency of nuclei showing early as well as late patterns of [^{3}H]thymidine labelling is higher in the treated glands; in this case the increase in discontinuous patterns in the treated unsynchronised glands would be more pronounced, since these patterns span a longer proportion of polytene S period (Steinemann, 1981).

Our results favour the latter possibility. The observed increase in the frequency of continuous type nuclei and decrease in the 3D and 2D type nuclei in chronically labelled treated glands in comparison to those in similarly labelled sister control glands shows that in the presence of ZR 515, many of the nuclei which were in late (3D or 2D) stages of a polytene replication cycle at the beginning of the culture, entered fresh cycles so

that they finally appeared to be continuously labelled, whereas in the absence of ZR 515, new cycles were not initiated in these nuclei in the control glands and they continued to show 3D or 2D patterns of labelling.

The results with synchronised cultures further support the stimulatory effect of the JH mimic on initiation of new polytene replication cycles. Earlier results of Achary et al. (1981) and our own data show that 5-FdUr can effectively block polytene nuclei in the intersynthetic period. However, upon release by culture in vitro, all these nuclei are not simultaneously released into the S period; rather this release occurs over a variable period of time. A higher frequency of the early patterns of labelling in the 5-FdUr-blocked glands cultured in vitro in the presence of ZR 515 than in the sister control glands, therefore, shows that within a given time a greater number of polytene nuclei are released than in its absence. The increase in the frequency of the discontinuous patterns in the later samples of the synchronised glands (Fig. 3) further shows that the new cycles stimulated by ZR 515 progress to completion in the normal manner.

It is to be pointed out that, unlike the synchronised glands, the stimulatory effect of ZR 515 in the unsynchronised glands does not become markedly apparent in the early samples. The control salivary glands from mid third instar larvae already show 70–80% labelling frequency in the early hours of in vitro culture, and since the number of nuclei in any gland remains constant, the stimulatory effect of ZR 515 will not be markedly obvious in these early samples. Moreover, as a sequel to the asynchrony in progression of polytene replication cycles in different nuclei of a gland, the new initiations stimulated by ZR 515 will be spread over a wider span of time. As a result of all these, only in the later samples does the stimulatory effect become progressively more obvious.

The above results appear to reflect the in vivo effects of JH on replication in polytene nuclei. As noted in the Introduction, the peaks of polytene replication in salivary gland nuclei during intermoult periods of larval life coincide with higher titers of JH in the haemolymph. Also, when *Drosophila* larval salivary glands are transplanted into the adult abdominal cavity where the JH titer is high (Riddiford and Truman, 1978), they continue polytene replication cycles beyond normal levels (Berendes and Holt, 1965). Recently, a stimulatory effect of JH on DNA synthesis in ovarian follicles of *Leucophaea maderae* (Koeppe and Wellman, 1980) and polyploid nuclei of the fat body in locusts (Nair et al., 1981) has also been shown. Thus, growth and DNA synthesis in endoreplicating cell types in a wide variety of insects may be influenced by JH.

The persistence of the same proportion of unlabelled and 1D type nuclei in the chronically labelled control and treated glands indicates that all polytene nuclei in a gland may not respond similarly to JH. In this context, we have also seen (data not presented) that in vitro treatment with ZR 515 fails to stimulate any new polytene replication cycle in salivary glands from older late third instar larvae. Comparable results were obtained by Berendes and Holt (1965) in their studies on transplantation of larval salivary glands into adult abdominal cavity. Thus, it seems that the stimulatory effect of JH on polytene replication cycles may also depend upon the developmental and physiological status of the target cell. These aspects need further study.

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